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FINAL REPORT

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CHEMICAL STUDIES OF ORGANIC TRACE METAL SUPPLEMENTS

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"Chemical Studies of Organic Trace Metal Supplements"

Final Report

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EXECUTIVE SUMMARY

This project is the fifth in a series of ADF projects aimed at understanding the chemical basis for copper deficiency in cows. This is a problem in some areas of Saskatchewan because of high sulfur and molybdenum levels in the diet. In the rumen, these can form ***thiomolybdates***, which bind copper strongly, forming an insoluble product and thus rendering the copper "unavailable", even although it is present in the diet.

This project has focussed on the development of a comprehensive computer simulation of the amounts of different chemical forms of copper (the "speciation") in the bovine rumen. In earlier studies, we developed a model which could predict the speciation, given the amounts of principal binding agents present (the volatile fatty acids, ammonia, etc.) and laboratory-based measurements of the different binding strengths of these to copper.

In our previous ADF project, we established the importance of copper binding by amino acids and small peptides. Although present in smaller amounts in the rumen than either VFAs or ammonia, they bind copper significantly more strongly. We also established that a particular class of peptides - those which mimic the N-terminus of Bovine Serum Albumin - might confer "protection" against thiomolybdates. Modern copper dietary supplements are often combined with peptides ("protein hydrolysates").

For all these reasons, we therefore developed a more sophisticated computer model to include copper-peptide species. This also necessitated measurement of many of the input data to the model (binding strengths of the various complexes formed). The model was validated by nuclear magnetic resonance measurements of both simulated bovine saliva and authentic rumen fluid samples, with additional amino acid and copper added. It was found that the model was reliable only if copper complexes involving binding to more than one binding agent at once ("mixed-ligand complexes") were included. The strengths of such complexes have not previously been the subject of much study; special techniques were developed and applied for their measurement in the laboratory.

The development of this model, combined with the insights previously gained about the chemical structures of the various complexes, will allow us both to predict probable protectants against thiomolybdate-based copper deficiency (whether resulting from the diet or administered as a supplement), and to test these in a comprehensive computer "model". This represents a significant advance in our ability to tackle this problem.

TECHNICAL REPORT

Introduction

In a continuing series of ADF-sponsored research projects, my group has been conducting fundamental research aimed at a better understanding of the ultimate chemical causes of bovine copper deficiency. Since this is a problem in many parts of this province, insight into why it occurs, and how we might protect against it, is of obvious importance. We believe that out of such basic science will emerge either nutritional strategies, or the development of more efficient, more "bioavailable" copper supplements.

The basic approach taken is to consider the fate of copper in the animal as a *chemical speciation* problem, determining in detail the different binding agents or *ligands* to which copper binds, the extent to which this happens, and the structures of the different species formed. Modern chemical analysis techniques allow such determinations to be made, even in very complex systems.

In early studies, we developed a computer "simulation" of the mix of copper species in the rumen. It was then possible to show, for instance, that the additional cost of "chelated" mineral supplements is not justified; these complexes do not persist in the rumen. The validity of such simulations was demonstrated by performing actual analyses of rumen content samples by nuclear magnetic resonance (NMR) spectroscopy.

However, it should be noted that such numerical simulations must necessarily represent a huge simplification of the true physiological situation, where many ligands, and metals, are present. The success of the approach rests on identifying the ***important*** constituents. Ligands present at very low concentration, or forming only very weak complexes, may be neglected without materially affecting the overall situation.

For this reason, further progress in speciation modeling was hampered by a lack of complete knowledge of what complexes might be important. Consequently, the last ADF project represented a "detour", into some very detailed studies of the chemical structures, rates of formation and strengths of various copper complexes to assess their importance. (The Principal Investigator is grateful for the continued support of the ADF in those somewhat "academic" studies).

It had been generally recognized that copper deficiency in Saskatchewan is not generally caused by insufficient copper in the diet, but by the presence of other substances, notably *thiomolybdates (TMs),* which can "tie up" the copper so that it cannot be utilised by the animal. By microbial action, sulfates (especially from well water) and other sulfur containing compounds can be reduced to sulfide, which in combination with feed molybdenum compounds in the rumen, form TMs. We needed to elucidate the details of this interaction, and of any hints in the chemical literature as to how it might be overcome.

First we developed a chemical synthesis of the various TMs in pure form, and a method of analyzing them to assess their purity, the first time that this had been accomplished. Then we performed a detailed study of TM stability and interactions with copper, employing various analytical techniques, such as UV/visible, NMR and mass spectroscopy, and X-ray crystallographic studies of the structures of the thiomolybdates.

One particular potentially protective interaction was of interest: it is known that copper forms a ***soluble*** complex with TMs in the presence of bovine serum albumin (BSA). Administering copper in the form of a Cu-peptide complex that binds TMs in a similar manner might allow better absorption of copper (II) by creating a soluble complex. We synthesized several small "model" peptides, which might mimic the binding behaviour of BSA itself and looked at their chemistry using NMR and UV/visible spectroscopy. It was confirmed that in the presence of BSA, the copper-thiomolybdate interaction could be slowed significantly, or even halted under some conditions. Most significantly, small protein fragments similar to the N-terminal region of the BSA protein chain - as small as two amino acids - seemed to confer the same advantage.

Aim of the present study

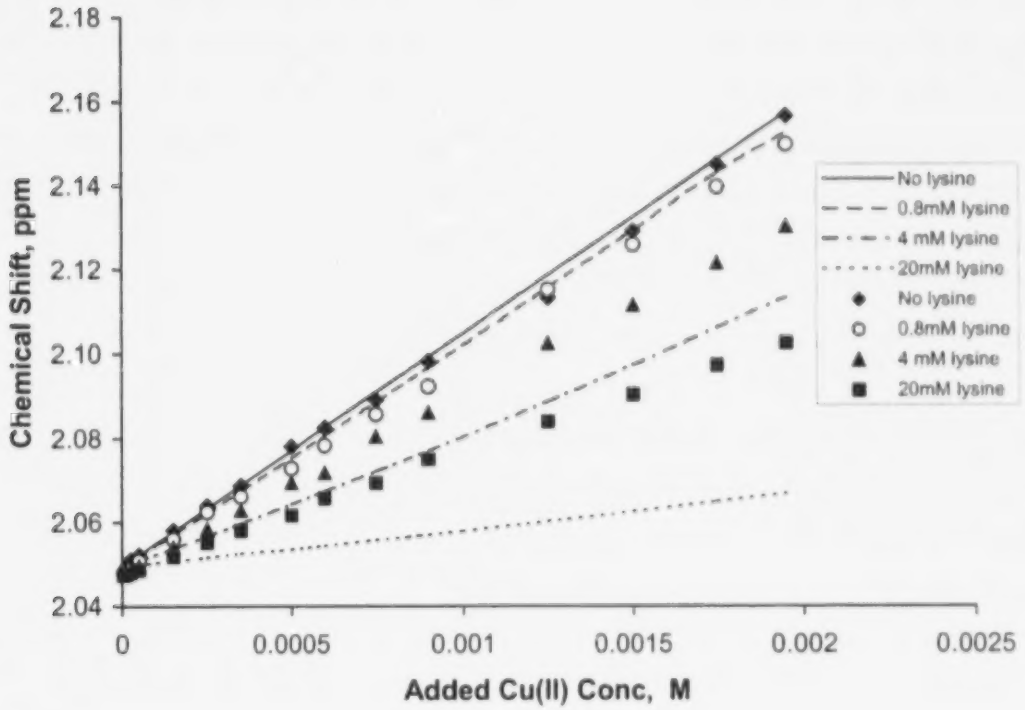
The above studies suggested strongly that the additional species which should be added to the "computer model" for copper speciation in the rumen were those involving amino acids and small peptides, especially those mimicking the N-terminus of BSA, for three reasons:
(1) Although they are present at lower concentrations than the Volatile Fatty Acids (VFAs) or ammonia in the rumen, we had established that their binding to copper was significantly stronger.
(2) If they are indeed potentially protectant against TM-based copper deficiency, their inclusion in the model, whether at normal (dietary) levels or enhanced (supplement) levels, is crucial.
(3) Some commercial copper supplements are "protein hydrolysates", ie. peptide mixtures.
Our main goal in this project was to succesfully include such species in our model.

Results and Discussion

It would be impractical to present (in a report of any reasonable length) ***all*** the results from this project. These comprise a huge amount of detailed chemical and numerical information, and much equally detailed interpretation of that information. What is presented here is a brief sampling of representative experiments, a mention of the rest, and a discussion of the significance of the whole.

Copies of three manuscripts prepared for publication in the scientific literature are appended; these should be consulted for more detailed information. A *complete* record of research findings will be held in the forthcoming M.Sc. thesis of Ms. Budu, and (in approximately two years' time) the Ph.D. thesis of Mr./Dr. Essilfie-Dughan. Copies of these will be forwarded to Dr. Javed as they become available. The Principal Investigator would, of course, be pleased to answer any enquiries about this research.

The first manuscript (***Speciation Modeling Of Copper(II) in the Bovine Rumen***, J. Essilfie–Dughan and R. S. Reid, found on page 14 of this report, details the modeling studies. The diagram below shows the success (or lack of it) of our previous modeling studies:



This shows the changes in the exact NMR frequency ("chemical shift") for the hydrogen atoms in acetate (one of the volatile fatty acids) as copper is gradually added, under various conditions, to a simulated rumen fluid sample (McDougall's solution plus typical amounts of the major products of rumen metabolism). The gradually increasing chemical shift is observed because an ever-increasing propotion of the acetate being studied is complexed to copper. If our computer "model" of the speciation is valid, we should be able to exactly predict (lines) the observed behaviour (symbols). In the case where no added Lysine is present (solid line, diamond symbols) this is the case. Where only a small amount (0.8 mM) of lysine is present, prediction is still adequate. However, higher amounts of added Lysine result in increasingly wild predictions. The overestimate of the flattening of the curve as more Lysine is added indicates that we are

overestimating the extent to which copper can "strip off" copper ion from acetate. This was unexpected, since we knew in great detail how strongly both the VFAs and Lysine complex xopper! Clearly, this model was not adequate, and it was necessary to find out why.

Considerable time was spent trying to adjust input parameters to account for the discrepancy; for instance, alteration of the assumed VFA concentrations over a wide range. However, this did not solve the problem. The VFAs do not complex copper very strongly (most is bound to ammonia in the rumen) and large variations both in overall VFA concentration and in the mix of VFAs had little effect. This in itself is significant; dietary strategies which simply alter VFAs are therefore unlikely to have any huge influence on copper bioavailablity.

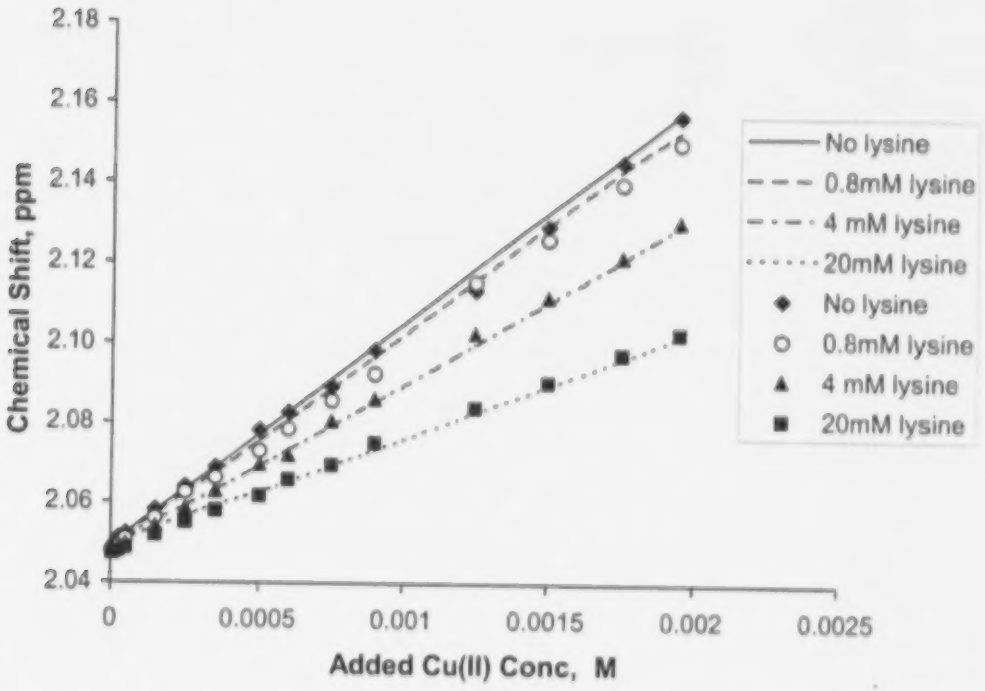
So what was wrong? The answer, ironically, lay in another corner of our laboratory.

Our extensive structural studies of copper complexes in the previous ADF project had confirmed the well-known tendency for copper (II) ions to form complexes with the ligand or ligands attached by four groups, with a preference for groups involving nitrogen atoms (amino groups, peptide amides, etc.). The more complex peptides, and proteins such as BSA, have four such groups. But many small molecules in solution, such as simple amino acids, have only two. And it is not clear how copper "jumps" from one situation to another. Losing all four ligand groups, then finding four new ones, is implausible - the former process would involve so much energy that it can readily be shown that it would occur far less often than is observed in practice.

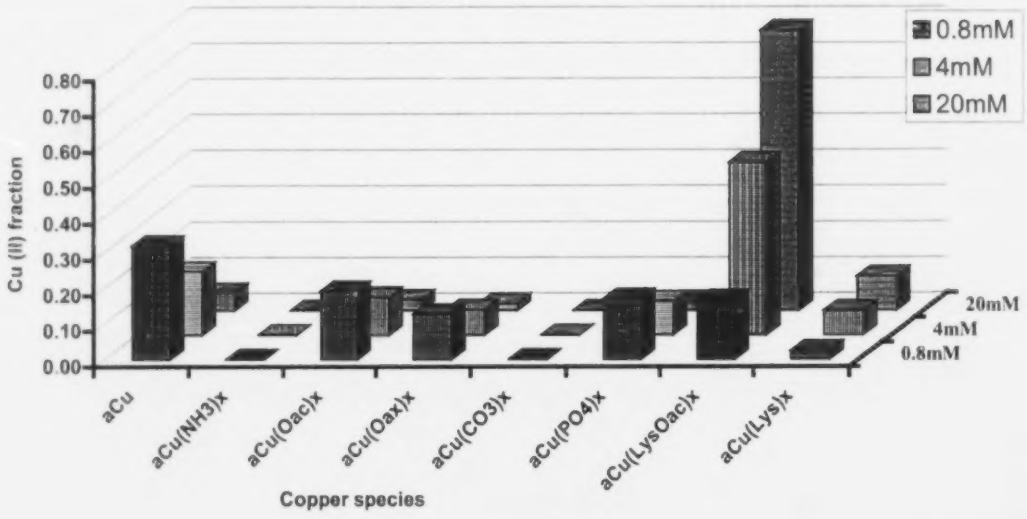
A more likely scenario is that the copper partially disengages from one complex, and partially forms the other, then withdraws completely from the original complex and fully forms the new one. At the halfway point, it is attached to both ligands, a structure called ***a mixed-ligand complex.***

Such systems are difficult to analyze, and little data is available. However, because such data was obviously relevant to predicting copper transport in general, Ms. Budu had already started developing techniques to measure the strengths of binding in mixed-ligand complexes.

So we decided to consider the possiblity of mixed-ligand complexes involving VFAs and Lysine in our modeling studies. Qualitatively, this accounts for the observed behaviour. Even although the Lysine does displace VFA from copper, it does so only partially - some VFA is still attached. So the curve does not flatten as much. When this was incorporated into the model, results improved dramatically:



The underlying reason can be seen in the figure overleaf. As the amount of added Lysine is increased, the fraction of the copper present as mixed-ligand complexes rises steadily. Clearly, models which do not include such species will become more and more inaccurate as added Lysine increases, exactly as observed.



The second manuscript (***NMR, UV-visible and Potentiometric studies of Proton and Cu(II) Complexation to His-Lys, Thr(Ac)-His-Lys and Asp-Thr(Ac)-His-Lys, Peptides Mimicking the NH_2-Terminal Cu(II)-Binding Site of Bovine Serum Albumin***, E. K. Quagraine, M. A. K. Budu and R. S. Reid, page 41) concludes the extremely detailed investigation of the structure and stability of small peptides which formed much of our last ADF project. The interested reader should peruse the manuscript for full details; basically, these studies confirmed that the copper complexes of these longer peptides have similar structures, and strengths, to those formed with smaller ones. This is significant, since it implies that even very small, simple peptides may have the "thiomolybdate protectant" properties of BSA.

We have always attempted to place our findings in a biological context wherever possible. The third manuscript (*Copper Dietary Supplements And Their Bioavailability In Ruminants and Other Animals,* E. K. Quagraine, M. A. Attaelmannan and R. S. Reid, page 72) is a "focussed review", interpreting animal science literature in the light of our recent findings. It does not present new data, so will not be discussed further here. However, a quick reading will illustrate the extent to which these "chemical" studies provide insight into biological problems.

STEPS TAKEN TO PUBLICIZE THIS WORK

Presentations:

Over the period of the project, Mr. Essilfie-Dughan, Dr. Quagraine or myself gave a total of five presentations (verbal or poster) of various aspects of this work, including presentations at the annual meetings of the Canadian Society for Chemistry, June 2001 and June 2002, and an invited seminar at the University of Manitoba (November 2002).

Publications:

Since this is academic research, the primary means of dissemination is via publication in the scientific literature. Out of the research performed as part of this project, five publications are planned. Copies of three manuscripts are appended to this report and are discussed above; a further two are in an earlier stage of preparation:

1. ***Speciation Modeling Of Copper(II) in the Bovine Rumen***, J. Essilfie–Dughan and R. S. Reid: to be submitted to the Journal of Inorganic Biochemistry.
2. ***NMR, UV-visible and Potentiometric studies of Proton and Cu(II) Complexation to His-Lys, Thr(Ac)-His-Lys and Asp-Thr(Ac)-His-Lys, Peptides Mimicking the NH_2-Terminal Cu(II)-Binding Site of Bovine Serum Albumin***, E. K. Quagraine, M. A. K. Budu and R. S. Reid: to be submitted to the Canadian Journal of Chemistry
3. ***Copper Dietary Supplements And Their Bioavailability In Ruminants And Other Animals***, E. K. Quagraine, M. A. Attaelmannan And R. S. Reid: to be submitted to the Canadian Journal of Animal Science.

A complete record of research findings will be held in the forthcoming M.Sc. thesis of Ms. Budu, and (in approximately two years' time) the Ph.D. thesis of Mr./Dr. Essilfie-Dughan. Copies of these will be forwarded to Dr. Javed as they become available.

POTENTIAL FOR COMMERCIALIZATION

This is fundamental research, so it is not necessarily expected that commercialization would result. However, the studies reported here provide important information towards recommendations for nutritional strategies which could protect against thiomolybdate-based copper deficiency.

We have gained an unprecedented level of knowledge of *both* (1) the structures of the various chemical species involved in this phenomenon (in our previous ADF project), and (2) a comprehensive, validated and trustworthy method of predicting their amounts in solution under a wide variety of conditions (in this project). Integration of these two studies in the next phase of this work (the subject of an upcoming ADF application) will result in further insight into how diet, and hence the mix of speciation-affecting metabolites, might affect the situation. Such recommendations may not be a marketable product, but they could nevertheless be of substantial benefit to Saskatchewan producers.

We also intend to integrate other small, simple peptides which mimic the BSA "thiomolybdate protectant" action into our computer model; identification of a simple, cheap substance with the desired properties might well yield a marketable compound, if such exists. We now have both a good idea of what such a substance might look like, and all the means to test the candidates.

PERSONNEL INVOLVED

Dr. Steve Reid devotes approximately 25% of his time to this work during the winter University term, and 60% over the summer months, principally via supervision of the students listed below. The students work approximately half-time on this work during the winter term, and full-time over the summer.

Emmanuel Quagraine completed his Ph.D. research in the group during the period of our previous ADF project (98000103-29BW), studying the solution chemistry of the thiomolybdates (TMs), and their interactions with Copper(II), by themselves and in the presence of other ligands, principally Bovine Serum Albumin (BSA). In a brief period of postdoctoral work, he completed his earlier work on the synthesis of BSA model peptide compounds, and studied their interactions with copper and the TMs.

Mildred Budu has substantially completed her M.Sc. research during the period of this project, performing the NMR and potentiometry-based solution studies of "mixed-ligand complexes" detailed above.

Joseph Essilfie-Dughan joined the group just before the start of this project, initially as an M.Sc. student. However, he plans to transfer to the Ph.D. program, and given his academic record and research productivity so far, I anticipate no difficulties. He has performed the detailed copper speciation modeling studies described above, including the adjustment to allow for the presence of "mixed-ligand complexes", and the NMR-based validation studies of the computer model using both McDougall's solution and authentic bovine rumen fluid.

It should be noted that both Dr. Quagraine and Ms. Budu, initially present in Canada on temporary student visas, have since applied (and received) Landed Immigrant status. Both intend to stay in Canada with their families, if possible in Saskatchewan. After a brief postdoctoral fellowship in the United States, Dr. Quagraine has returned to Canada

since the conclusion of this project, and is currently working in Saskatoon on a Federal Government research contract held by myself, and as a sessional lecturer at the University of Saskatchewan. It is good to know that ADF-sponsored research can help alleviate, or even reverse, the much-discussed "brain drain".

Prof. Heinz-Bernhard Kraatz of the Chemistry Department assisted Dr. Quagraine in the peptide syntheses, and allowed use of his equipment. We thank Department of Animal and Poultry Science staff for access to a fistulated cow for collection of rumen fluid samples. Various other technical personnel have been involved; thanks are due in particular to **Mr. Ken Thoms**, instrument technician, for assistance with elemental analysis; and **Dr. Keith Brown**, of the Department of Chemistry, who runs the Nuclear Magnetic Resonance facilities. None of these people billed the project for their time, although contributing both personal and instrument time to it.

STATEMENT OF EXPENDITURES

A final statement for the project has been submitted previously by the University of Saskatchewan. All expenditures were in compliance with ADF guidelines. The project came in under budget at the end of the contract period, due to Dr. Quagraine's leaving from his postdoctoral position in the group somewhat prematurely, due to a tempting offer from a U.S. researcher.

(Manuscript to be submitted to J. Inorg. Biochem.)

SPECIATION MODELING OF COPPER(II) IN THE BOVINE RUMEN

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ABSTRACT

Copper bioavailability in the bovine is intimately related to the particular metal ion species that exist in different body compartments of the bovine. Thus computer simulation and solution speciation studies has been used in a previous study to examine this assertion in bovine saliva and the rumen. Results indicated a good agreement between the computer simulation and the experimental values for the bovine saliva. However some discrepancy was between the computer simulation and the experimental values for the rumen. The present study aims at exploring the reasons for the discrepancy and refining the computer simulation to agree with the experimental values. As it was previously done, ^{1}H NMR experiments and computer simulation was used in this study. Results show a good agreement between the computer simulation and experimental values upon introduction of previously unaccounted for copper (II) mixed –ligand species.

KEY WORDS

Copper (II) speciation, Computer simulation, ^{1}H NMR, Bovine rumen

We have previously reported studies of the metal-ligand speciation of copper-lysine ('chelated' copper) and copper chloride (inorganic copper) in McDougall's solution (simulated ruminant saliva) using both computer simulations [1] and NMR experiments [2]. These are two widely used copper supplements for ruminants, Speciation studies in bovine saliva are important because this is the first biofluid encountered in the gastrointestinal (GI) tract.

The study was also extended to the rumen (the first of the four-chambered stomach of ruminants). A similar approach to the McDougall's solution studies was employed; computer simulation was used to predict the copper speciation, followed by NMR studies of biological sample. Agreement between the two sets of results was found to be satisfactory. Some discrepancy was found between the model and predicted values as lysine was introduced into the model. That is at higher concentration of lysine, there was increasing deviation between the predicted and experimental values[2]

The present study aims at refining the speciation modeling of Cu (II) ions in the bovine rumen by investigating the cause of the discrepancy between computer model and experimental results as lysine is added as an exogenous ligand and correcting the discrepancy. The essence of adding excess lysine is to enhance bioavailability and also to test the validity of the model. This is because changes the concentrations of metals or ligands will result in the establishment of a new equilibrium position. If the model mimics the behaviour of the system, it should correctly predict the effect of adding new ligands to the mixture. Computer simulations and NMR experiments of a laboratory simulated rumen fluid was used in the study.

2. MATERIALS AND METHODS

2.1 Chemicals, Materials, Reagents

All solutions used in this study were prepared using doubly distilled water which was degassed before use. All reagents used were of analytical grade and were used as purchased without any further modifications. Ammonia solution and sodium hydrogen carbonate were obtained from British Drug House (BDH) Chemicals, Toronto; Acetic acid Potassium Chloride from EM Science (Affiliate of MERCK); Propanoic acid from Fischer Scientific Company, Fair Lawn, NJ, U.S.A.; Butanoic acid from Aldrich Chemical Company, Milwaukee, WI, U.S.A.; Sodium hydrogen phosphate and L-lysine from Sigma - Aldrich company, St Louis, MO, U.S.A.; and Copper (II) chloride from Allied Chemicals, NY, U.S.A.

2.2 ^{1}H NMR experiments

Solution mixtures containing concentrations of ligands used in the rumen model and various copper (II) concentrations were prepared in 100 ml volumetric flasks. The ionic strength was adjusted to 0.15 **M** using various concentrations of potassium chloride. 10 ml aliquots of the prepared solutions were put into labelled vials and the pH of each solution was adjusted to a pH of 4.00 (± 0.01) by the addition of concentrated solutions of KOH and HCl using a syringe.

0.4 ml of each of the solution (with pH adjusted) was put into labelled NMR tubes and 0.05 ml of Dimethyl-2- silapentane-5-sulfonate (D.S.S.) (50%) was added to each tube as an internal standard. A ^{1}H NMR experiment was conducted to obtain the chemical shift of the methyl proton of acetate on each solution using the bionnomial '1-3-3-1' pulse sequence technique for water suppression on a Bruker AM300 spectrometer operating at a frequency of 300 MHz (proton).

2.3 pH Measurements

All pH measurements were made with an Accu-pHast standard combination glass body pH electrode (Fischer Catalogue No. 13-60-297) and an Accumet pH meter. The electrode was calibrated by standard U.S. National Institute of Standards and Technology (N.I.S.T.) approved methods, using a set of primary standard solutions whose pH values have been accurately established by N.I.S.T [3].

2.4 Calculations

The simulation model included the major end products of rumen metabolism as potential copper ligands. These are mostly the short chain volatile fatty acids, acetic, propanoic and butanoic, together with ammonia as well as the bicarbonate and phosphate moieties from the saliva. It is difficult to lay down exact figures for concentration of the ligands in the rumen, as these are highly dependent on the diet fed to the ruminant. Nevertheless, average values could be

used after comparison with literature values [4, 5, 6, 7, 8] under typical conditions. Table 1 shows typical analytical concentrations of the ligands

Table 1: Values of ligand concentrations used in the rumen modeling studies

In setting up the model use was made of complex formation constants that has been measured previously in the group [1] or by careful selection from literature. Table 2 shows the set of formation constants used in the model while table 3 gives the formation constants of lysine species used in the computer simulation . Where literature constants were obtained at temperatures other than the temperature of the rumen, the Van't Hoff equation [9] was used to correct them. Values that were obtained at ionic strengths other than the ionic strength of the rumen, were corrected using the Davies equation [10], and compared with those obtained by the IUPAC Specific Ion interaction Theory program.

The concentration of copper used for the model was 2.5×10^{-5} **M**, a modest value for the nutritional requirements of a healthy cow [11]. The concentrations of the VFAs were varied in different studies. These are known to vary substantially depending on the diet fed to the ruminant. For instance, diets high in concentrates tend to produce fluids that are high in acetic acid but low in propanoic acid, whereas forage diets are known to increase the level of propanoic acid.

The chemical problem is put in an algebraic form using the law of mass action. The mathematical model is chosen on the basis of the known chemistry of the system, using a series

of equations to define the system. These equations are then used to generate numerical values for concentrations of the species formed, which are consistent with the known values of the relevant formation constants. At the same time, the sum of each entity (metal or ligand) should also add up to the known total concentration for that entity in all forms.

The resulting equations are coded into a Microsoft Excel® spreadsheet, a powerful numerical analysis package. Calculations were performed using an IBM compatible PC 1.4 GHz (intel Pentium IV) Gateway microcomputer. One of the advantages of using a spreadsheet is that one can watch the value at each iteration. Observing progress towards convergence makes the method more understandable, and often reveals some of the subtleties involved.

The general algorithm involves the assumption of equilibrium concentrations of input species , calculation of all other species formed, summation of all species and comparing it with original total concentrations. Based on the agreement of actual total concentrations and those returned from the calculations, the estimates of the assumed concentrations are adjusted, the worst estimate at each iteration being selected for adjustment.

The addition of each new ligand to the model results in one more mass balance equation. Each species is expressed as the product of a set of chemical components that define the equilibrium position and a formation constant. With each new ligand the expression for the total copper concentration changes to accommodate the new set of species.

When the species have settled at equilibrium, the dissociation and formation constants of the various species are recalculated and compared with the input values to check for discrepancy. This helps ensure that, the results obtained from the model are indeed reliable.

Calculations were performed at average ruminal pH of 6.3 and then from a pH of 5.8 to 6.8, to ensure that the simulation covers normal range of ruminal pH. Also VFA levels are known to vary substantially depending on the diet fed to the ruminant. For instance, diets high in concentrates tend to produce fluids that are high in acetic acid but low in propanoic acid, whereas forage diets are known to increase the level of propanoic acid [5]. Speciation calculations were therefore performed under varying concentrations of VFAs.

Table 2: Formation constants ($\log \beta = [M_pL_qH_r]/[M]^p[L]^q[H]^r$) data used in simulation. All values were obtained or corrected to a temperature of 38° C and ionic strength of 0.15 M

Table 3 The formation constants of lysine species used in the computer simulation. . All values were obtained or corrected to a temperature of 38° C and ionic strength of 0.15 M

3. RESULTS AND DISCUSSION

3.1 Computer Simulations

Results from the simulation (Figure 1) indicate that the bulk of copper is present as carbonate, phosphate and acetate at the lowest normal ruminal pH of 5.8. As the pH increases within the normal ruminal pH range, the copper acetate species fall off as the copper carbonate

and the copper phosphate species dominate up to the highest normal ruminal pH of 6.8 where copper exists mostly as carbonate complexes. Only a small fraction of the total copper concentration is attached to the rest of the volatile fatty acids – propanoic, butanoic, isobutanoic, pentanoic and isopentanoic, and a tiny fraction attached to ammonia.

Also according to the model copper is virtually completely bound in the rumen fluid, with only trace amounts of "free" ionic copper existing. This is in agreement with other studies that report an immeasurable amount of ionic copper in biological fluids. This is directly attributed to the high complexing ability of the copper (II) ions.

At the average ruminal pH of 6.3, 60.4% of the copper exist as carbonate, 23.8% as phosphate, 10.3% as acetate, 3.8% as the other volatile acids, 1.4% as "free" ionic copper and 0.2% as ammonia. Figure 1 illustrates the distribution of copper amongst the LMM ligands of rumen at a pH of 6.3. $aCu(CO_3)x$ represent the fraction of all copper carbonate species; $aCu(PO_4)x$, all copper phospate species; $aCu(NH_3)x$, all copper amino species; aCu, "free" ionic copper; aCu(Oac)x, all copper acetate species; and Cu(Oax)x, fraction of all copper complexes of the other volatile fatty acids present in the rumen. The changes that occur with varying pH values are shown in Figure 2. It is apparent that the overall speciation is little affected by the changes in the pH values that the rumen experiences.

The predominant acid in the rumen is acetic acid. Roughage diets high in cellulose give rise to acid mixtures particularly high in acetic acid. As the proportion of concentrates in the diet is increased, the proportion of acetic acid falls and that of propanoic acid rises. It is known

that some absorption of volatile fatty acids does in fact take place in the rumen. If the copper is complexed to these fatty acids, it might well be that some copper absorption takes place even prior to its arrival at the small intestine,

Figure 1 Distribution of copper amongst the LMM of rumen at a pH of 6.3

Figure 2 Distribution of copper amongst the LMM of rumen as pH is varied between 5.8 and 6.8.

where most of the absorption is thought to take place. However the amount of copper complexes to these acids is minimal and thus the role the copper-VFA complexes play is unlikely to be significant. It is concluded that the proportions of the VFA produced would not markedly affect copper absorption.

Calculations performed under varying concentrations of VFAs. did not result in any significant changes in the overall distribution of copper (II), as shown in figure 3.

Figure3 Distribution of copper amongst LMM ligands in rumen as concentration of VFAs are varied at a pH of 6.3

The speciation of copper among the LMM of the rumen was not altered when copper was introduced into the model as 1:1 copper lysine supplement.as shown in figure 4. The copper carbonate species are still the dominant fraction copper species with copper amino species being the least fraction of copper species present.

Figure 4 Distribution of Copper-lysinate among LMM of rumen at pH of 6.3

3.2 Validation of Rumen Model.

As mentioned in the introduction, computer simulation results are necessarily incomplete and describe only those aspects that are considered relevant. It therefore becomes necessary to obtain some assurance that the model is a correct representation of the equilibrium in the model. These tests would involve the comparison of calculated and experimentally determined values of some parameter.

The parameter employed here is the ^{1}H NMR chemical shift of the acetate CH_3 protons. The chemical shifts of acetate can be calculated using the model, as most of the input data is already available. The data input required are the pH, concentration of the different components, and chemical shifts of acetate species. A number of test solutions were used to test the validity of the computer model. These test solutions were made up of simulated rumen liquor spiked with varying levels of Cu(II) concentration.

The chemical shift of acetate was calculated with varying levels of Cu(II) concentration added to the test solutions from the results of the computer simulation. The observed chemical shift is given by

$$\alpha_{obs} = \alpha_A\delta_A + \alpha_{HA}\delta_{HA} + \alpha_{CuAx}\delta_{CuAx} \qquad (1)$$

where α_{obs} is the observed chemical shift; α_A, α_{HA}, α_{CuAx} fractions of acetate, acetic acid and all different copper acetate complexes respectively; and δ_A, δ_{HA}, δ_{CuAx}, chemical shifts of acetate, acetic acid and all different copper acetate complexes respectively.

The increased amounts of Cu are predicted to produce a consistent increase in the chemical shift of acetate as the Cu levels is increased. This is explained by the increasing fraction of the Cu- acetate complexes. This is not surprising considering the high chemical shift of the composite acetate copper complex 20.4 ppm, (Table 4). Using simulated solution sample containing all the components in the computer model, NMR experiments were actually carried out to measure the changes in chemical shifts of acetate. The experiments were conducted at a temperature of 25°C , ionic strength of 0.15M and pH of 4.0. It was decided to work at a pH of 4.0 to minimise Cu hydrolysis, and to provide a basis for direct comparison with previous studies. [2]

Table 4: Calculated chemical shifts for acetate species used in study[2]

Experimental results obtained showed a good agreement with model predictions as shown in figure 4.

Figure 5. Chemical shift of methyl protons of acetic acid as a function of copper (II) concentration in simulated rumen fluid Curve shows predicted values and points (■) show experimental values

The computer model also predicts that the addition of 0.8 m**M** lysine would not alter the speciation significantly (figure 2 & 4). This is due to the low concentration of the added lysine compared with the concentration of other ligands present (Table1) . When actual NMR experiments were conducted this was also the case. The model closely predicts the observed changes in chemical shift. Small deviations are the result of experimental errors in the NMR, and show no systematic trend away from the predicted line. .

The computer model predicts a further change in the speciation picture when 4.0 m**M** lysine is added. Figure 5 illustrates changes in chemical shift of methyl protons of acetate as function of copper (II) concentration as different amount of lysine are added.. With the higher concentration of lysine, and considering the strength of the Cu- lysine complexes, lysine would bind more of the Cu atoms. This reduces the amount of Cu-acetate complexes, and hence a downward shift is noted for the acetate shift.

However, when these predictions are compared to the data obtained from an actual NMR experiments, some discrepancy is noted between the model and the experimental values. Reasons for this will be discussed below.

When 20 m**M** of lysine were added, the model predicts that the bulk of the copper will now be present as copper lysine complexes. The effect on the acetate chemical shift is similar to that at 4.0 m**M** added lysine, but more pronounced. However, experimental data shows little change in the chemical shift of acetate with the increasing levels of copper. The discrepancy between model and experiment is now pronounced.

The implication of this result is that the model does not adequately explain the speciation at this level of lysine. The model seems to predict a lower amount of Cu-acetate complexes than that actually observed. The model underestimates the level of complexation between lysine and Cu, it appears as though there is more lysine complexing the Cu than is actually present.

Figure 6. Changes in chemical shift of methyl protons of acetate with different amount of lysine as a function of copper (II) concentration in simulated rumen fluid. Points represent experimental data, while curves represent model predictions

The extent of the discrepancy is more pronounced with increasing levels of copper. This means that the computer model could not adequately predict the experimental data observed with increasing lysine concentration.

There are a number of reasons that could possibly account for this discrepancy. The discrepancy could be as a result of using values for the metal concentrations that do not accurately reflect those appearing in the model. To explore this possibility, the levels of metals in the fluid were measured using Inductively Coupled Plasma Atomic Emission Spectroscopy. The results obtained showed some differences from metal concentration values appearing in the literature. This however did not appreciably alter the results obtained from the model . This had a minimal effect on the chemical shift variation with added copper levels.[12] Another possible explanation for the incongruity is that it is due to some interaction of lysine with some species in the rumen liquor. To this NMR experiments were conducted for increasing lysine concentration in the simulated rumen fluid and the results did not show any variation in the

chemical shift of the various components of the rumen fluid. Thus a possible a possible interaction of lysine with any of the species in the rumen fluid was ruled out. The discrepancy could also be due to lack of knowledge of all possible complexes that could form. To address the issue of discrepancy in the present study, the possibility of formation of mixed ligand complexes was explored since formation of mixed ligands are very common in biofluids.[13] After a thorough literature study, mixed ligand complex formation involving Cu(II) and some of the ligands present in the rumen were explored. These mixed ligands include copper- amino-acetate ($CuNH_3OAc$), copper-propanoate-acetate (CuOPaOAc), copper-lysinate-acetate (CuLOAc). The mixed ligand species and their formation constants, estimated from their 1:1 copper complex formation constants by calculation, (16) were introduced into the computer model one after the other. The mixed ligand complex formation involving acetate and lysine introduced into the computer model, gave a very good fit to the experimental data as shown figure 7.

This means that as lysine is introduced into the rumen fluid, it is unable to strip copper off the acetate but rather form mixed ligand complex with it and that this complex which was previously unaccounted for in the computer model also contributes to overall chemical shift of the acetate complex accounting for the discrepancies observe between the experimental data and the model.

Figure 7 Changes in chemical shift of methyl protons of acetate with different amount of lysine as a fraction of copper(II) concentration. Points represent experimental data, while curves represent model predictions.

Speciation calculations were then performed at a pH of 4.0 for varying concentrations of lysine. The results of these calculations are shown in figure 7.For a lysine concentration of 0.8 m**M**, only a small fraction of Cu (II) exists as the species that was not accounted for in the previous model, mixed ligand complex (i.e. CuLOAc), and that is why there was no clearly observed deviation between experimental and predicted chemical shift of methyl protons of acetate (figure 6). However as the concentration of lysine increases, the mixed ligand complex becomes the major species and this accounts for the deviation between predicted and experimental chemical shift in the previous model (figure 6). About 80% of copper existed as the mixed ligand complex when the concentration of lysine was increased to 20 m**M**; this explains the still more pronounced deviation between the predicted and experimental chemical shift.

Figure 8 Distribution of copper amongst LMM ligands of rumen and varied concentration of lysine at a pH of 4.0

4. CONCLUSIONS

This study forms part of our continuing investigation into the efficacy of complexed mineral dietary supplements using a computer – based speciation simulation models and NMR spectroscopic techniques. Results presented here indicate that mixed-ligand complexes must be included if the speciation is to be succesfully modeled. Recognizing the paucity of mixed-ligand formation constant data in the literature, we have also embarked on a program measuring such constants for relevant copper-carboxylic acid-amino acid systems.

ACKNOWLEDGEMENTS

Financial support from the Agriculture Development Fund (ADF) of the Province of Saskatchewan, Canada, and from the University of Saskatchewan in the form of a Graduate Scholarship for Joseph Essilfie-Dughan, is gratefully acknowledged. We also acknowledge Dr. M. A. Attaelmannan for his pioneering work in this study.

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TABLES

Table 1: Values of ligand concentrations used in the rumen modeling studies

Ligand	**Concentration** (mM)
Acetate	84
Propionate	21
Butyrate	13
i-Butyrate	1
Valerate	0.7
i-Valerate	0.3
PO_4	20
HCO_3	21
Ammonia	11

Table 2: Formation constants ($\log \beta = [M_pL_qH_r]/[M]^p[L]^q[H]^r$) data used in simulation. All values were obtained or corrected to a temperature of 38° C and ionic strength of 0.15 M

System	p	q	r	log β	Ref.
Acetic Acid	0	1	1	4.74	[14,15]
Copper Acetic Acid	1	1	0	1.60	[14,15]
	1	2	0	1.1	
	1	3	0	0.3	
	1	4	0	-0.1	
Propanoic Acid	0	1	1	4.68	[14,15]
Copper Propanoic Acid	1	1	0	1.70	[14,15]
	1	2	0	0.92	
Butanoic Acid	0	1	1	4.62	[14,15]
Copper Butanoic Acid		1	1	0	1.75	[14,15]
	1	2	0	0.95	
iso-Butanoic Acid	0	1	1	4.62	[14,15]
Copper iso-Butanoic Acid	1	1	0	1.75	estimate
1	2	0	0.95		
Valeric Acid	0	1	1	4.62	[14,15]
Copper Valerate System	1	1	0	1.75	
	1	2	0	0.95	
Phosphoric Acid	0	1	1	1.7	[14,15]
	0	1	2	6.46	
	0	1	3	10.79	
The Copper Phosphate	1	1	0	3.3	[14,15]
	1	1	1	1.3	
Carbonic Acid	0	1	1	6.02	[14,15]
	0	1	2	9.57	
The Copper Carbonate	1	1	0	6.75	[14,15]
	1	2	0	3.17	
Ammonia	0	1	-1	9.14	[14,15]
Copper Ammonia	1	1	1	4.1	[14,15]
	1	2	0	3.51	
	1	3	0	2.88	
	1	4	0	2.09	

Table 3 The formation constants of lysine species used in the computer simulation. . All values were obtained or corrected to a temperature of 38° C and ionic strength of 0.15 M

System	p	q	r	log β	Ref.
Lysine	0	1	1	10.529	[1,2]
	0	1	2	19.658	
	0	1	3	21.914	
Copper (II) Lysine	1	1	1	18.81	[1,2]
	1	2	2	35.90	
	1	2	1	26.41	
	1	2	0	15.73	
	1	1	2	23.58	

Table 4: Calculated chemical shifts for acetate species used in study[2]

Species		Chemical Shift, ppm
Acetic acid	A	1.903
	HA	2.076
Copper acetate	[a]CuA_X	20.40

[a]CuL_X refer to all the different copper acetate complexes.

FIGURES

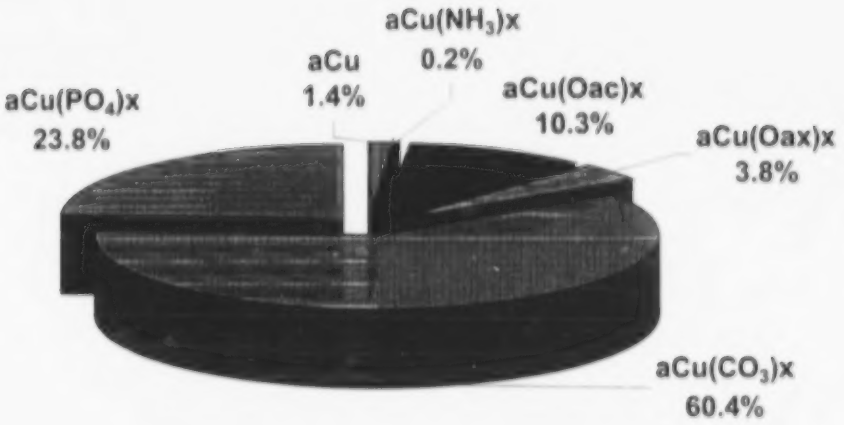

Figure 1 Distribution of copper amongst the LMM of rumen at a pH of 6.3

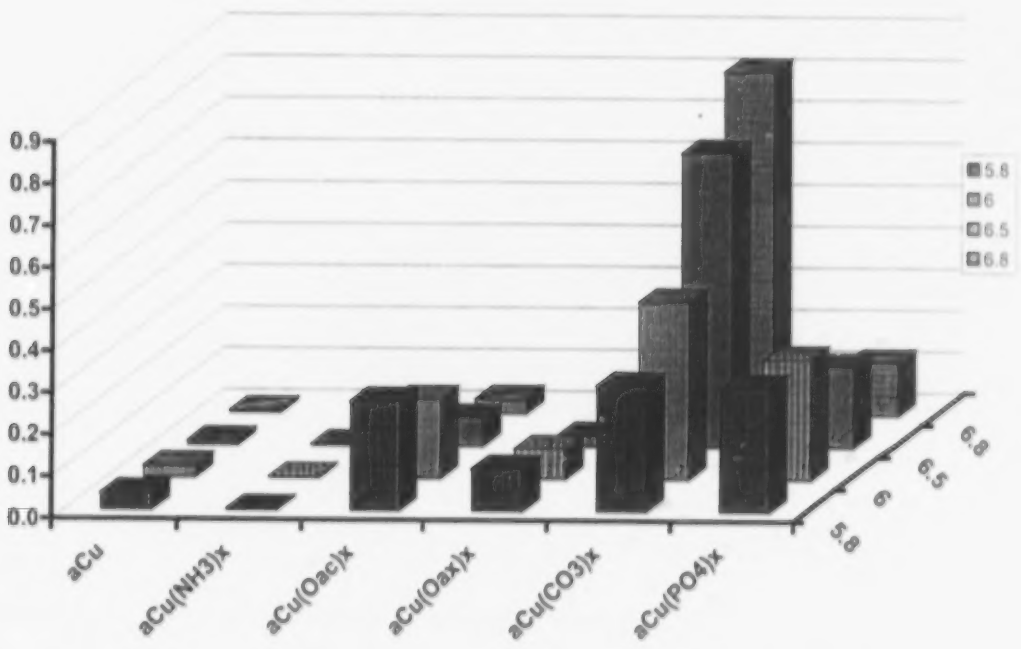

Figure 2 Distribution of copper amongst the LMM of rumen as pH is varied between 5.8 and 6.8.

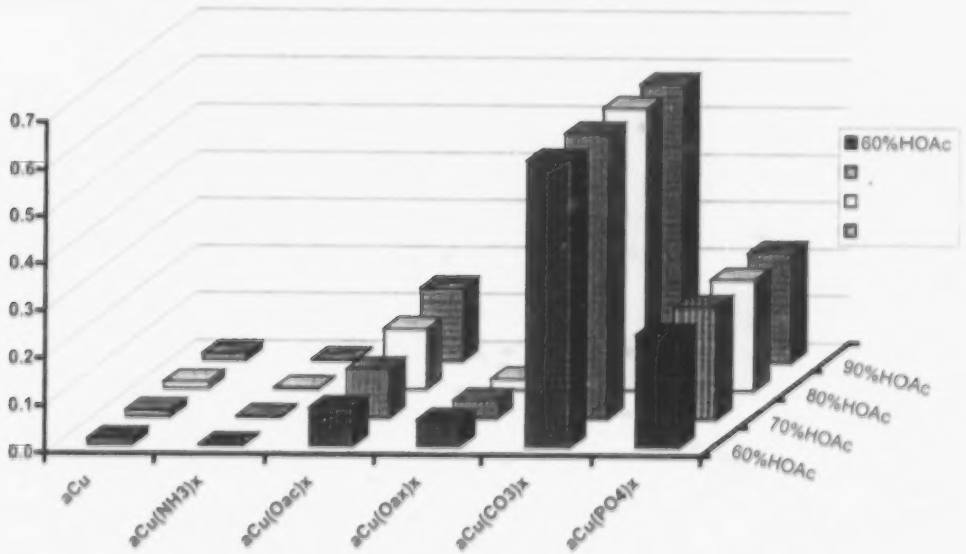

Figure3 Distribution of copper amongst LMM ligands in rumen as concentration of VFAs are varied at a pH of 6.3 .

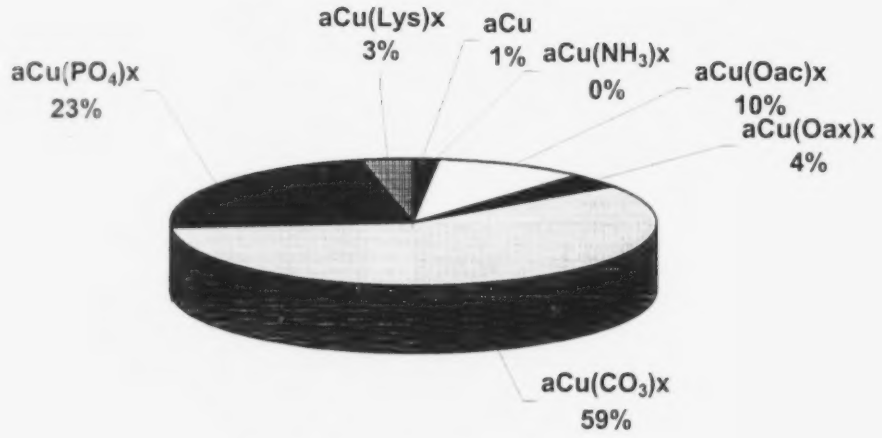

Figure 4 Distribution of Copper-lysinate among LMM of rumen at pH of 6.3

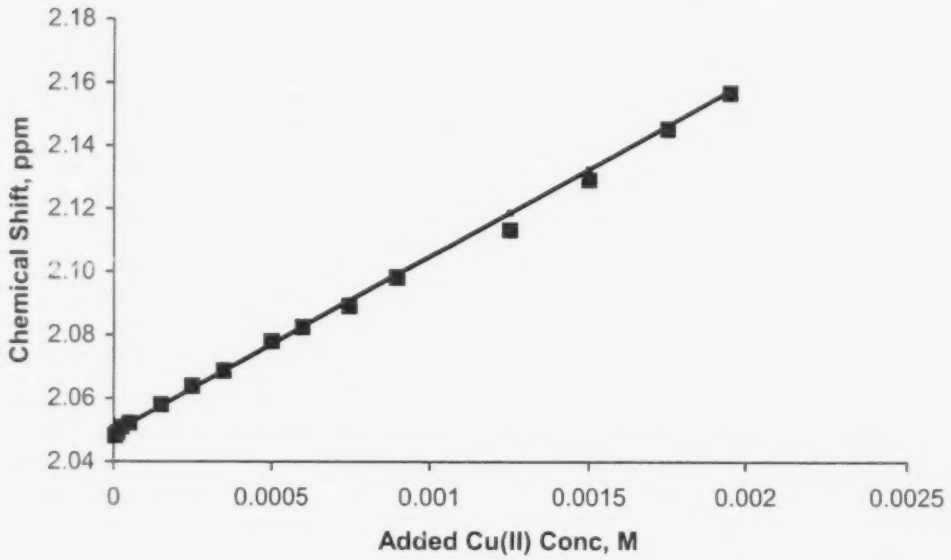

Figure 5. Chemical shift of methyl protons of acetic acid as a function of copper (II) concentration in simulated rumen fluid Curve shows predicted values and points (■) show experimental values.

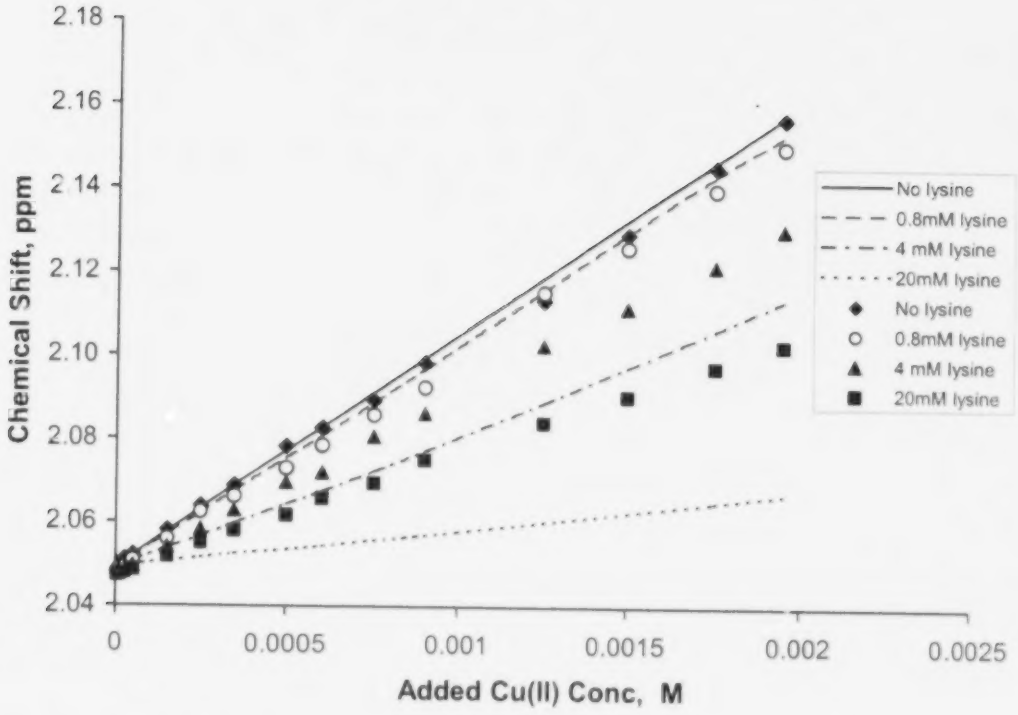

Figure 6. Changes in chemical shift of methyl protons of acetate with different amount of lysine as a function of copper (II) concentration in simulated rumen fluid. Points represent experimental data, while curves represent model predictions

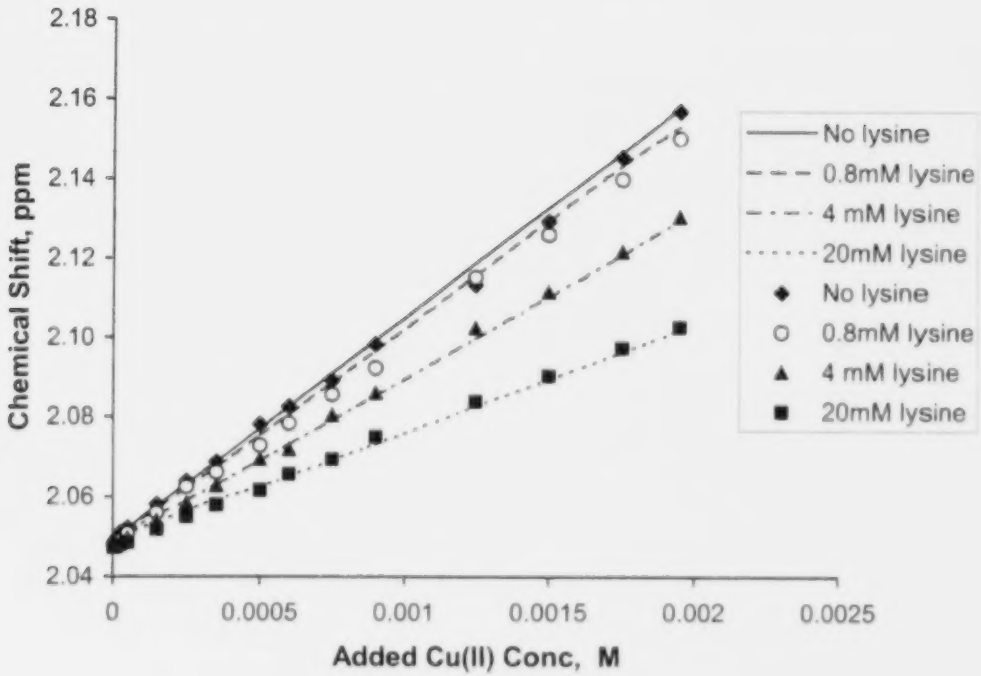

Figure 7 Changes in chemical shift of methyl protons of acetate with different amount of lysine as a fraction of copper(II) concentration. Points represent experimental data, while curves represent model predictions.

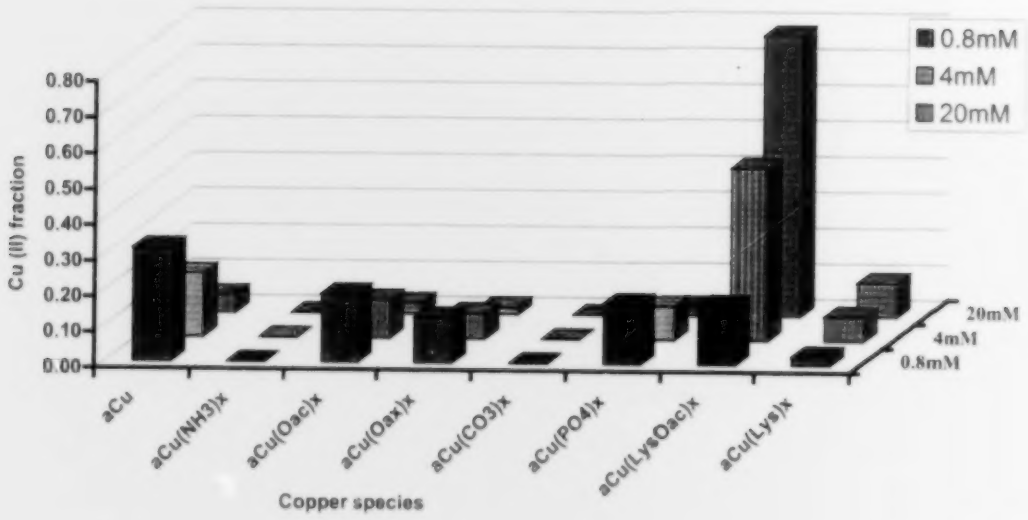

Figure 8 Distribution of copper amongst LMM ligands of rumen and varied concentration of lysine at a pH of 4.0.

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NMR, UV-visible and Potentiometric studies of Proton and Cu(II) Complexation to His-Lys, Thr(Ac)-His-Lys and Asp-Thr(Ac)-His-Lys, Peptides Mimicking the NH_2-Terminal Cu(II)-Binding Site of Bovine Serum Albumin

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1. Introduction:

Proton and metal interactions with peptides is a subject of the utmost interest in research because of their biological and clinical implications. The important physiological implications of Cu-albumin complexation for the transport of Cu(II) in the blood [1] make it of interest to investigate the structures and the stabilities of the complex species in solution.

Asp-Thr-His-Lys sequence is known to constitute the N-terminal region of the protein Bovine Serum Albumin (BSA) and has been implicated to provide a strong binding site for Cu(II) ions [2]. Differences in the N-terminal of various serum albumin affect their binding ability to Cu(II). BSA, with an N- terminus amino acid sequence of Asp-Thr-His-Lys and various other serum albumins such as Human Serum Albumin, HSA (Asp-Ala-His-Lys) and Rat Serum Albumin, RSA (Glu-Ala-His-Lys), whose first four amino terminal sequence are in parenthesis, bind strongly to Cu(II). Others like Canine Serum Albumin, CSA (Glu-Al-Tyr-Lys) and Pig Serum Albumin, PSA (Asp-Thr-Tyr-Lys) have little or no ability to bind Cu(II). The difference in affinity of the N-termini of these two sets of serum albumins for Cu(II) is attributed to a conserved His at the third position from the α-amino terminal (i.e. His-3) in BSA, HSA and RSA effecting strong binding to Cu(II) [3,4]. Unlike these serum albumins, CSA and PSA have a tyrosine residue in the third position in place of the His residue [3,4]. These serum albumins therefore lose their potential to bind Cu(II) as strongly as the His-3 containing albumins [5]. Many literature reports also indicate that the position of histidine residue in a peptide chain is critical with regard to the speciation and the structure of the Cu(II)-complexes [6-8].

Based primarily on Cu(II) complexation studies with isolated 24-residue polypeptide chain from the peptic digestion of BSA, earlier reports [2,9] also suggested the involvement of the α-amino nitrogen atom of Asp, the intervening peptide bond nitrogen atoms of Asp-Thr-His and the imidazole nitrogen atom (N-3) [2,9,10]. A square planar Cu(II)-BSA complex was proposed [9]. Similarly, early reports on studies to investigate the binding of Cu(II) to HSA and its peptide models led to the same hypothesized coordination mode as suggested for BSA [11-14]. The techniques used in these old studies were predominantly potentiometry, and visible and optical rotatory dispersion spectroscopy.

However, some later studies with HSA peptide models have pointed also to the involvement of the O of the Asp β-carboxylate side chain, in addition to the four nitrogen atoms above [3,15], giving a penta-coordinated structure involving the four N atoms in a square planar coordination to Cu(II) and an axial coordination from the β-carboxylate O of Asp. The techniques used in these studies were 1–D ^{1}H and ^{13}C NMR spectroscopy. It is noteworthy that NMR spectroscopy provides more information on structures of the complexes as well as binding sites than potentiometry and visible spectroscopy, which were used in the older studies above.

A more recent study on the binding of Cu(II) to both HSA and BSA, using both 1-D and 2-D ^{1}H NMR also led to a proposed square planar coordination with ligands of α-NH_2 of Asp-1, the two deprotonated NHs of Ala-2 (HSA) or Thr-2 (BSA) and His-3 and the imidazole of His-3 [16]. In addition, the results suggested a conformation where the Lys side chain lies over the His ring in the Cu-albumin complexes in both cases, but did not indicate the involvement of the β-carboxylate group in the coordination. Recent studies of Cu(II) binding to BSA peptide models, as performed in the case of HSA, are absent from the literature, and thus the issue as to whether or not the β- carboxylate group is also involved in BSA coordination to Cu(II) is still a matter of conjecture.

Understanding the role played by BSA in the transport of Cu(II) in blood requires detailed solution chemistry studies of the structures and the stabilities of the complexes formed in aqueous solution. The use of small model peptides has proven valuable in elucidating the structural features contributing to the formation of such metallocomplexes [9,17]. A good BSA model peptide for Cu(II) complexation should mimic the N-terminal amino acid sequence and in the least, the peptides used as models should contain the conserved His-Lys sequence for albumins exhibiting high binding affinity for Cu(II).

Apart from being good models for the study of the interactions of Cu(II) with proteins, small His-Lys-containing peptide, e.g. Gly-His-Lys have been known to have some specific biological activities such as altering the growth rate and survival of cultured hepatoma cells and hypotocytes [18]. These activities are enhanced when they are complexed to Cu(II) and other transition metals [18]. Structure–function studies revealed that several tripeptides with a His-Lys linkage were nearly as active as Gly-His-Lys [19]. It has been shown that Gly-His-Lys stimulates collagen synthesis and hence serves as an activator for wound repair [20]. Complexes

of such peptides may also be involved in the exchange and transport mechanism of trace Cu ions [19] in animals.

Although some compilations about the acid dissociation constants (K_a's), stability constants (K_f's) and affinities to Cu(II) ions of different His-Lys containing peptides have been published [1,6,18,21-24], results from different authors often disagree to an extent that cannot simply be attributed to factors such as temperature. For instance, Lau and Sarkar [24] and May et al. [18] have both studied and reported the proton and Cu(II) complexation of Gly-His-Lys at 25°C, I = 0.15 **M** NaCl and 37°C, I = 0.15 **M** NaCl respectively. However the identity and the number of species and the formation constants determined in these two studies differ significantly from each other to an extent which cannot be attributed to the difference in temperature. Lau and Sarkar's study was based on potentiometric method, complemented by spectrophotometric and equilibrium dialysis methods. May et al. also used the potentiometric method for their study.

Although potentiometric methods are more sensitive and provide precise macroscopic constants, they do not provide as much information as NMR spectroscopy. NMR spectroscopy provides detailed structural information [15] and may be used to determine microconstants as well. The existence, approximate concentrations, structure of different species can be determined by direct observation. This tool has commonly been used for the determination of complex structures when the metal ion is diamagnetic (e.g. [25]) but rarely for paramagnetic ions like Cu(II). This is attributable to lack of sensitivity arising from the line broadening resulting from complexation of the paramagnetic species. However, advantage can sometimes be taken of this line broadening to obtain valuable structural information [15, 26-29].

We recently reported [30] the synthesis of His-Lys and Thr(Ac)-His-Lys and their acid-base behavior using ^{1}H NMR. Preliminary Cu(II) complexation studies of these peptides by the same technique were also reported. In this present paper, pH-metric and UV-visible spectrophotometry have been used to complement the ^{1}H NMR studies of these peptides to obtain more reliable formation constants of their Cu-complexes and also to come to a more unequivocal conclusion about their structure and their speciation. Also, by using all the three techniques, the present paper extends the study to the His-Lys containing tetrapeptide, Asp-Thr(Ac)-His-Lys as a closer model to mimic Cu(II) binding to BSA.

2. Experimental:

2.1 Materials and Methods

All solvents and reagents were used as received without further purification. The solvents and reagents were obtained as follows: KH_2PO_4, $NaHCO_3$, $CuSO_4$ and anisole (Fisher Scientific); KNO_3, $MgSO_4$, and NaOH (BDH chemicals); Na_3PO_4 (Sigma) and EDC (Calbiochem- Advanced ChemTech respectively); HOBt, HBr (30 wt % solution in acetic acid), 99% TFA, $(C_2H_5)_3N$, and charcoal (Aldrich); and DSS (Merck, Sharp and Dohme). The deuterated solvents deuterium oxide (D_2O, 99.9%) and chloroform ($CDCl_3$, 99.8%) were obtained from Cambridge Isotope Laboratories. The protected amino acids were also obtained as follows: Boc-His(DNP)-OH.isopropanol (Calbiochem-Novabiochem), and H-Lys(Z).HCl, Z-Asp(OBzl)-OH and Boc-Thr(Bzl) (Calbiochem, Advanced ChemTech). The peptides His-Lys and Thr(Ac)-His-Lys were synthesized as described previously [30]. All abbreviations are defined below.

HPLC analyses, Mass spectra and C, H and N analyses were performed as previously described [30]. NMR measurements were performed on a Bruker AM 300 spectrometer at frequencies of 300.13 MHz and 75.47 MHz for ^1H and ^{13}C NMR, respectively. Further details of the NMR measurements were described in a previous paper [30]. UV/Visible spectra were recorded with an S2000 Ocean Optics Inc. spectrometer over the range 370-800 nm, using 1 cm cuvettes.

2.2 Asp-Thr(Ac)-His-Lys Synthesis

The synthesis of Asp-Thr(Ac)-His-Lys is outlined in Scheme I. Procedures for the peptide synthesis were similar to those outlined earlier [30].

2.2.1. Preparation of Z-Asp-Thr(Bzl)-His(DNP)-Lys(Z)-OBzl (B)

The compound A (scheme I) was synthesized and purified as described in [30]. After Boc-deprotection of A (see scheme I) with TFA, it was coupled to Z-Asp(OBzl)-OH with HOBt/EDC. Pure product (B) was obtained in 82% yield by the same process as described

before [30]. The purity was assessed by TLC, using the solvent mixture 90:8:2 $CHCl_3$:MeOH:AcOH as the eluant (R_f = 0.58). HPLC analysis (CH_3CN /H_2O gradient) showed a single peak. Molecular mass (g mol^{-1}) for $C_{49}H_{56}N_8O_{13}$. Calc. 1204:24 found (FAB mass spectroscopy) 1204.46. ^1H NMR (δ in ppm, $CHCl_3$): 8.78(1H, dd, J_{HH} = 1.8 Hz, 10.6 Hz, aromatic H of DNP), 8.43-8.50(1H, m), aromatic H of DNP), 8.26(1H,d, J_{NH} = 7.4 Hz, NH of His), 8.16 (1H, d, J_{NH} = 8.5 Hz, NH of Asp) 7.57(1H, d, J_{HH} = 8.8 Hz, aromatic H of DNP), 7.19-7.47 (26H, 25 aromatic H of Ph ar.d 1H imidazole), 6.77 (1H, s, imidazole), 6.57 (1H,d, J_{NH} = 6.5 Hz, NH of Thr), 5.92 (1H,d, J_{NH}= 8.7 Hz, NH of Lys), 5.03-5.19 (9H, m, overlapping signals of 4(OCH_2Ph) and 1H, Z-Lys NH), 4.86 (2H, m, α-CH of His and α-CH of Asp), 4.43-4.63 (4H, m, overlapping signal of 1(OCH_2Ph), α-CH of Lys, α-CH of Thr), 4.27 (1H, β-CH of Thr), 2.92-3.24 (6H, m, overlapping signals of β-CH_2 of His, β -CH_2 of Asp and ε -CH_2 of Lys), 1.57-1.62 (2H, m, β-CH_2 of Lys), 1.34-1.37 (2H, m, γ-CH_2 of Lys), 1.09-1.27(5H, m, overlapping signals of δ-CH_2 of Lys) and γ-CH_3 of Thr). ^{13}C NMR (δ in ppm, $CDCl_3$): 171.9, 171.8, 171.1, 170.8, 169.8, 156.6, 156.3 (7C=O), 146.7 (s, quaternary C of DNP), 143.9 (s, quaternary C of DNP (C-NO_2)), 140.4, 138.4,136.9,136.1, 135.7, 135.4, 135.2 (7 ternary aromatic C), 128.7-127.6 (overlapping signals of 27 methine aromatic carbons), 121.6 (methine aromatic C), 117.8(methine aromatic C), 117.6 (methine aromatic C), 74.4 (methine β-C of Thr), 74.0 (methine C), 71.5, 67.7, 67.3, 67.1, 66.6 (5 methylene C of OCH_2Ph), 52.9 (methine C), 52.4 (methine C) and 51.6 (methine C), 40.9 (methylene C), 36.2 (methylene C), 31.7 (methylene C) , 29.3 (methylene C), 28.7 (methylene C), and 22.6 (methylene C) and 16.6 (CH_3 of Thr).

2.2.2. Preparation of Asp-Thr(Ac)-His-Lys (D)

. Simultaneous removal of the DNP and Z protecting groups of B was accomplished as described earlier [30] to obtain the solid C. The compound was purified by column chromatography (R_f = 0.6, $CHCl_3$: MeOH, 2:1) to obtain a yield of 51%. The three Bzl protecting groups of compound C were removed by HBr/AcOH treatment. During the treatment, an acetyl-protecting group was introduced to the Thr side chain to give compound D. The purification of the compound was the same as described earlier [30] to obtain a yield of 60%. HPLC analysis (CH_3CN /H_2O gradient) showed a single peak. Molecular mass (g mol^{-1}) for

$C_{22}H_{35}N_7O_9$: Calc. 541.56; found (FAB mass spectroscopy) 541.29. Elemental Analysis based on the formula: $[C_{22}H_{41}N_8O_9Br_3]$.1/3 CH_3OH.2/5 CH_3COOH. The CH_3 signals of CH_3OH and CH_3OOH were both observed in the ^{1}H-NMR spectrum in D_2O and H_2O and their actual values determined by spreadsheet calculations. Calc. C, 33.23; H, 5.29; N, 13.42. Found C, 33.31; H, 5.15; N, 13.82. ^{1}H NMR (δ in ppm, D_2O): 8.62 (1H,d, J_{HH} = 1.2 Hz, imidazole) 7.28 (1H, s, imidazole), 5.30 (1H, doublet of quartet, J_{HH} = 3.5 Hz, J_{HH} = 6.5 Hz, β-C*H* of Thr), 4.68-7.73 (1H, α-C*H* of Asp and α-C*H* of His overlapping with residual H_2O signal), 4.48 (1H, d, J_{HH} = 3.5 Hz, α-C*H* of Thr), 4.28 (1H, dd, J_{HH} = 2.5, J_{HH} = 6.0 Hz, α-C*H* of Lys), 3.32-3.09 (4H, m, overlapping signals of β-CH_2 of His and Asp), 2.99 (2H, t, ε-CH_2 of Lys, J_{HH} = 7.6 Hz), 2.03 (3H, s, CH_3C=O(Thr)), 1.88-1.63 (4H, m, overlapping signals of β-CH_2 of Lys and δ-CH_2 of Lys), 1.49-1.34 (2H, m, γ-CH_2 of Lys), 1.26 (3H, d, J_{HH}= 6.5 Hz, γ-CH_3 of Thr). The amide NH, aromatic NH, and the ammonium protons were detected in water (See Fig. 5 (i)) as follows: ^{1}H NMR (δ in ppm, in H_2O) 8.75 (1H, d, J_{NH} = 7.7 Hz NH of Lys,) 8.42 (1H, d, J_{NH} = 7.6 Hz NH of His), 8.37 (1H, d, J_{NH} = 6.5 Hz, NH of Thr), 7.69 (1H, bs, N1-H(Im), 7.54 (ε-NH_3^+ of Lys) and 7.13 (1H, N3-H(Im)). ^{13}C NMR (δ in ppm, D_2O) 178.8, 177.3, 175.4, 173.8, 173.7(5C=O), 136.1, (tertiary C imidazole), 130.9 (methine C of the imidazole), 119.8 (methine C imidazole), 72.7, 59.6, 56.0, 54.8
(4 methine C), 2 (41.7), 33.0, 2 (28.8), 24.7 (6 methylene C), 23.0 (*C*H_3 of -OCO*C*H_3), 19.0 (γ-CH_3 of Thr).

2.3 UV-visible spectroscopic studies

The spectra were recorded on varying the pH from ~ 3 to ~12 of a 1:1 Cu(II):peptide reaction mixture. The concentrations of the peptides His-Lys, Thr(Ac)-His-Lys, and Asp-Thr(Ac)-His-Lys used were respectively 6.6 mM, 12.0 mM and
3.0 mM. The pH adjustment was achieved by adding small quantities (a few μL) of concentrated NaOH (between 2 and 10 M) dropwise with micropipettes to about 3.5 mL of the 1:1 Cu(II)-peptide mixture. The use of the concentrated NaOH minimizes the error due to dilution.

2.4 ^{1}H NMR experiments in aqueous solutions

All aqueous solutions used in this study were prepared using deionized and distilled water. Solution preparation and pH measurements were carried out at 25 ± 1°C. Ionic strengths were adjusted to 0.2 M using KNO_3. All pH measurements were performed as described previously [28,30], with the exception of the fact that measurements in this present study were done directly in the 5mm NMR tubes with a MI-412 micro-combination pH probe (Microelectrodes, Inc. USA). Where necessary, pH adjustments were performed by the addition of concentrated solutions of NaOH and HNO_3 using a Hamilton microliter® syringe, so that the effect of dilution could be neglected. A phosphate buffer (pH 7.0 and ionic strength 0.2 M) was prepared from Na_2HPO_4 and KH_2PO_4 [30]. Additions of Cu(II) ions were made with aliquots of stock solutions of Cu(II) sulfates (0.2 M) in 0.5M KNO_3.

All calculations and data analysis were performed as described previously [30].

2.5 pH potentiometric studies

All solutions were prepared using doubly deionized and degassed distilled water. A "carbonate free" 0.0971 M NaOH solution adjusted to 0.2 M with KCl was used as the titrant. The computer program GLEE (glass electrode evaluation) was used to calibrate the glass electrode and to estimate the carbonate contamination of the base (Typically, < 0.8% carbonate impurity was obtained). This program was also used and to obtain the pseudo-Nernstian standard potential, slope of the electrode, and the concentration of the base and pK_w, which were used as, input data in HYPERQUAD potentiometric data files. Formation constants for complexes with H^+ and Cu^{2+} were calculated from titration curves carried out in 2.2-2.4 ml peptide samples in concentration range 1-2 x 10^{-3} M at ligand to Cu(II) ion ratio of 1:1 and 2:1 and in the pH range 2 -11.3 (or till a precipitate or opalescence was observed in solution). At least three titration curves were obtained for each titration system. HCl was used to adjust the initial pH value when needed. The number of experimental data points varied from ~60 to ~150 per titration. All pH-metric measurements were carried out at 25 ± 1 °C using a water bath equipped with a Haake D1 circulator (Germany) for thermostatting the solutions, which were of a constant ionic strength of 0.2 M KCl.

The potentiometric titrations were carried out using an automated computer controlled gravimetric titrator that had previously been developed and modified in our laboratory

[31,32,33]. The current version of the auto-titrator used in this study, is based on the Lab-View graphic program, which allows the control of the titrant delivery from the reservoir (via valves) using input parameters. Once the computer is instructed to begin the titration, it monitors the emf (E) or pH as well as the weight of the titrant added until the final pH target is attained. Details of the architecture and operation of the titrator can be found in a thesis submitted to the University of Saskatchewan [33]. The weights of the titrant were input directly into the HYPERQUAD program as independent variables and the E values as the dependent variables.

3. Results and Discussion

3.1. Acid–Base Behavior of Asp-Thr(Ac)-His-Lys by ^{1}H NMR

Fig. 1 shows ^{1}H NMR chemical shift data for a 9.8 mM solution of Asp-Thr(Ac)-His-Lys as a function of pH. As previously explained [30] for the peptides His-Lys and Thr(Ac)-His-Lys, the equilibria involved in this peptide are also fast on the NMR time scale. Even though at higher pH, the exchange is, predictably, somewhat slower, as shown by the significant line broadening observed, it is still in the fast-exchange regimen. The observed chemical shift δ_{obs} is therefore the average of the chemical shifts of the individual Asp-Thr(Ac)-His-Lys species, weighted by their relative amounts [28,30]; i.e.

$$\delta_{obs} = \delta_{H_4L}\alpha_{H_4L} + \delta_{H_3L}\alpha_{H_3L} + \delta_{H_2L}\alpha_{H_2L} + \delta_{HL}\alpha_{HL} + \delta_L\alpha_L$$

δ_x is the chemical shift of a given species x, and α its mole fraction. L refers to the "fully deprotonated" Asp-Thr(Ac)-His-Lys species as shown in Figs. 2 and 3. (Deprotonation of the N-1(Im) group can be neglected since that occurs at extremely high pH. The fifth protonation, to form an H_5L species (deprotonation of the C-terminal COOH), similarly occurs only at low pH. Over a physiologically relevant pH range, α for both these is effectively zero.) HL refers to singly protonated peptide. See Fig. 2 for details of the ionization scheme assumed for Asp-Thr(Ac)-His-Lys in solution. For reasons of clarity, charges have been omitted from the equations. By defining the acid dissociation constants K_{a1}, K_{a2}, K_{a3} and K_{a4} in the usual manner, it can be shown that:

$$\alpha_{H_4L} = H^4 / D;\ \alpha_{H_3L} = K_{a1}H^3 / D;\ \alpha_{H_2L} = K_{a1}K_{a2}H^2 / D;$$
$$\alpha_{HL} = K_{a1}K_{a2}K_{a3}H / D;\ \alpha_{L} = K_{a1}K_2K_3K_{a4} / D$$

where

$D = (H^4 + H^3.K_{a1} + H^2.K_{a1}.K_{a2} + H.K_{a1}.K_{a2}.K_{a3} + K_{a1}.K_{a2}.K_{a3}\ K_{a4})$ and

$H = \text{antilog}(-pH) \sim a_{H^+}$

By performing non-linear regression analysis of the data, as previously described [30], the chemical shifts for the individual species as well as the values of K_{a1},K_{a2}, K_{a3} and K_{a4} can be determined.

The high quality of the resultant fit of predicted to experimental values can be judged from Fig.1. Such analysis was performed for each chemical shift curve, except, where overlap made this impossible. See Fig. 3 for the K_a values thus obtained. Also shown in parentheses are the changes in the chemical shifts observed during the ionization process governed by a given pK_a. The size of this change indicates the sensitivity with which the observed NMR chemical shift "senses" the ionization process [30].

The chemical shift changes for the imidazole CH protons (Figs 1 and 3) clearly indicate that these are "sensing" ionization at the imidazole N3 ($pK_{a2} \sim 6.5$). This process was also "sensed" by the β-CH_2 of His and the NH of Im N1, except that the sizes of the chemical shift changes involved are different, in the manner expected. The NH of Im N1 and the β-CH_2 of His sense the protonation of the N-terminal amino group ($pK_{a3} \sim 8.7$), although the sensitivity of the latter is very small. A third protonation is "sensed" by the NH of Im N1 at a lower pKa value of (~5.0). This process is more sensed by CH_3 protons of the acetyl derivative of the Thr residue. The most likely protonation site is that of the β-COOH of Asp. Since these protons (CH_3s) are several bonds away from the ionization site, this suggests a "through-space" effect. The same reason accounts for how NH- Im(N1), several bonds way from this site, senses this process. This suggests a conformation where the Asp and the Thr side chains lie in close proximity. The chemical shift changes observed for the pK_{a4} process ($pK_{a4} \sim 10.6$) increase down the Lys side chain (from β to ε positions) as also observed for His-Lys and Thr(Ac)-His-Lys [30], confirming that this is the protonated Lys ε-NH_2 ionization.

Averaged values for pK_{a1}, pK_{a2}, pK_{a3} and pK_{a4}, weighted by the magnitude of the chemical shift change in each case, are 4.91 ± 0.06, 6.55 ± 0.03, and 8.74 ± 0.06, 10.66 ± 0.04 respectively. The dominant species under physiological pH are therefore H_3L and H_2L. Both of these species have the terminal amino group protonated. It is only beyond pH 8 that the fraction of the HL species (possessing a deprotonated terminal amino group) is significant.

The pK_a for the ionization of the β-carboxylate is somewhat higher than those reported for most Asp containing peptides, which have typical pKa values < 4 [34]. There are however a few longer peptide chains with pK_a values > 4; for example, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe has COOH (Asp) pK_a= 4.67 [35].

3.2. Potentiometric determination of pKa's of the Peptides

The pK_a values obtained for the peptides His-Lys, Thr(Ac)-His-Lys and Asp-Thr(Ac)-His-Lys in this study by potentiometry are shown in Table 1. These are provided alongside other values previously reported in the literature, and the values obtained in this study by NMR for comparison.

The most basic group of the His-Lys is the ε-NH_2 group of Lys residue. Its protonation constant 10.34 determined by potentiometry in this study falls between a critically selected value of 10.09 [34] determined by ^{1}H-NMR, in D_2O solution of ionic strength 0.1 M at 37°C [25] and a value of 10.59, previously obtained by us, in aqueous (H_2O) solution of ionic strength 0.2 M at 25°C [30]. The difference of about 0.15 pKa units as compared to that obtained previously [36] also by potentiometry and at the same temperature may be attributable, in part, to differences in ionic strength. The adjustment of the value to an ionic strength of 0.1 M using the Specific Interaction Theory (SIT) software [37] gives a closer value of 10.41 in comparison to the previously determined value by potentiometry. The pK_a value for the deprotonation of the α-NH_2 proton obtained by potentiometry in this study is in close agreement to that obtained under similar conditions using ^{1}H-NMR [30] and also compares very well to others in the literature (Table 1). The pK_a values for the deprotonation of the imidazole proton obtained by potentiometry in this study is however significantly lower than the previously reported value by the same technique, although closer to that determined by NMR. The pKa for the fourth

protonated species (H_4L) seem to be less than 2 and its inclusion in the model, using the Hyperquad program, worsened the statistical parameters and was rejected as being negative or excessive by the program.

Apart from our previous reports, there are no other reports in the literature on the protonation of Thr-His-Lys or Asp-Thr-His-Lys for direct comparison. Comparison is therefore being made between the potentiometric data for these peptides with those by ^{1}H-NMR from our previous report [30] and this current study. As shown in Table 1, the potentiometric data are more precise than the NMR data. Considering the imprecision of the NMR data, there seem to be a reasonable matching between the two methods for the deprotonation constants of NH_2 (ε-Lys) and NH_2 (α-Thr) of Thr(Ac)-His-Lys and for NH_2 (ε-Lys), NH (Im), and COO^-(β-Asp) of Asp-Thr(Ac)-His-Lys. The pK_as for the deprotonation of imidazole NH of the tri-peptide and the deprotonation of the α-NH_2 of the tetra-peptide obtained by the two methods, however, differ significantly (~ 0.7 pK_a units in each case (Table 1)). It is uncertain at this moment, the cause of this discrepancies but the possibility that some of the measured constants by NMR are micro-constants can not be completely overruled. It is not uncommon to find such discrepancies sometimes between Potentiometric and NMR data on dissociation constants of peptides, where different deprotonation steps may significantly overlap with each other. For instance, the deprotonation constant (macroconstant) for the β-COOH obtained for the tetrapeptide His-Val-Gly-Asp was estimated as 4.27 by potentiometric method but as 3.27 by NMR [46].

3.2.1 Comparison of pK_a values of Asp-Thr(Ac)-His-Lys to those of His-Lys and Thr(Ac)-His- Lys.

Numerous pK_a values for His-containing dipeptides [6,7,38], tripeptide [7,18,24,38] and tetrapeptide or higher chain peptides [7,8,39,40] have been reported. See Table 2. Reports for small peptides containing the His-Lys chain seem to be confined to the obvious dipeptide His-Lys [25,34] and the tripeptide Gly-His-Lys [7,18,24, 25,34 38,43].

Since even minor changes in the amino sequence can lead to significant changes in the acid-base behavior of a peptide it is difficult to make any meaningful comparison with pK_as of other peptides in literature. Noteworthy, however, are the significant differences between the pK_a's (as determined by potentiometry for instance) for the protonated α-amino group in the dipeptide His-Lys (~7.1), the tripeptide Thr(Ac)-His-Lys (~7.0) and that observed for the tetrapeptide Asp-Thr(Ac)-His-Lys (~ 9.5), which would certainly influence their relative metal binding abilities. The lower pK_a value for the dipeptide as compared to the tetrapeptide may therefore be expected, due to a stronger interaction between the amino group and the adjacent imidazole group, as compared to the interaction between the β-carboxylate and the amino group. The imidazole and the terminal carboxylate groups are too remote from the amino group to interact significantly with it. The lower pK_a observed for the protonated α-amino group of the tripeptide than both the dipeptide and tetrapeptide cannot be similarly explained, since in the absence of any group in close proximity to the α-amino for any meaningful interaction, the pK_a should rather increase. Perhaps the tripeptide adopts a conformation that favors the deprotonation of the α-ammonium proton.

There are also differences in the pK_as, as exemplified with the NMR data of the protonated Im N3 groups for His-Lys (~ 5.7) and Thr(Ac)-His-Lys (~ 6.3) and that of Asp-Thr(Ac)-His-Lys (~ 6.6). It appears from these data and those obtained by potentiometry (Table 1) that the pk_a of the Im N3 bears some relationship to its position from the α-amino site. The closer it is to this site the lower the pK_a. This trend seems to be the case in most instances in literature (Table 2). Generally, when the His residue is at the position 1, the pk_a ranges are < 5.7; at position 2 the pk_a ranges are 5.6-6.8; at position 3, 6.6-6.8, and for positions 4 and beyond pk_a

ranges > 6.8 are observed. Although there seems to be some overlaps in some few cases, especially the positions 2 and 3, this could serve as a basis for the estimation of pK(Im N3).

As shown in Table 1, the pK_as obtained by both potentiometry and ^1H NMR for ε-Lys deprotonation for Asp-Thr(Ac)-His-Lys is not substantially different from those of His-Lys and Thr(Ac)-His-Lys.

3.3. Potentiometric studies of the Cu(II)-peptide Complex System

3.3.1 Cu(II)-His-Lys

The stability constants of the Cu(II) complexes of His-Lys determined are reported in Table 3 alongside with two other sets of values previously reported in the literature [36,37] for comparison. The corresponding distribution diagrams for the data in the present study are shown in Fig. 4. Although the data in this study and those of the two previously reported, in general, concur reasonably, there are some differences in the number and type of species determined in each case. In the two previous reports, both conducted in aqueous solution of ionic strength 0.1 M, the monomeric species (1 1 1) was detected in contrast to the dimeric species (2 2 1) with log β = 42.86 identified in this study at 0.2 M ionic strength. We also detected the monomeric species (1 1 0) with log β = 12.79, but [36] could not detect this monomer but instead the dimer (2 2 -1). In the present study also, Hyperquad seemed to suggest the presence of both the monomeric species (1 1 1) and the dimeric species (2 2 -1) but the concentration of these species appeared low for accurate calculation of the log β. Their inclusion in the models employed in the hyperquad program, if log β values around those obtained by the previous authors were used as input data, improved the statistical parameters (chi-squared and sigma) slightly, but showed then as negative. As shown in Fig.4b, for a ligand to metal ratio of 2, the bis-complex 1 2 2 dominates the other species, within the pH range 5 to 9. The presence of the excess ligand, prevents the formation of the dimeric species detected in the equimolar solutions of the peptide and the metal ion.

3.3.2 Cu(II)-Thr(Ac)-His-Lys

The stability constants of the Cu(II) complexes of Thr(Ac)-His-Lys are summarized in Table 4, whereas Figs. 5 a and b show the distributions of species for the tripeptide as a function of pH in solutions of ligand to metal ratios of 1 and 2, respectively. Comparison of the stability constants data with those of Gly-His-Lys [24] show the presence of the same types of species, with the exception of a 1 2 -2 species, which was not detected for Thr(Ac)-His-Lys in this study but reported for the Gly-His-Lys analogue. The formation constants for the Cu(II) complexes of Thr(Ac)-His-Lys (Table 4), are typically higher than those of the Gly-His-Lys by about 1.2 to 1.4 log β units . The 1 2 0 species of the Cu(II)-Thr(Ac)-His-Lys complex is only 0.4 log β units higher than the Gly-His-Lys analogue, whereas the 1 1 0 and the 1 2 2 of Thr(Ac)-His-Lys is higher by 2.1 and 3.3 log β units.

It is clear from Fig. 5a that the major species for the equimolar solution of the metal and the ligand, in the wide pH range of 4.5 to 9.7, which encompasses the pH of physiological relevance, is the 1 1 0 species. However, in the presence of excess of the ligand, as shown in Fig. 5b, almost the whole amount of the Cu(II) metal ion is bound in the 1 2 2 species in the wide pH range of ~2 to ~ 8, while the 1 1 0 species is only significantly detected, amongst others, above pH 8.

3.3.3 Cu(II)-Asp-Thr(Ac)-His-Lys

Table 4 also shows the complex-formation constants of the tetrapeptide with the Cu(II) ion. Six species were detected in the pH range explored; all of them a mono-complexes with different degree of protonation. Fig. 6 shows the distributions of species for the equimolar Cu(II)-tetrapeptide complex as a function of pH. As shown by the figure, coordination starts at pH under 4 with the formation of the 1 1 3 species as the major species. Deprotonation of this species occurs with increase of pH to form the 1 1 2 species as the dominant species at pH ~ 5. The 1 1 2 species is deprotonated and replaced, in dominance, by the 1 1 1 species with an increase of pH by only 0.5 unit. At physiological pH the species 1 1 0 is the major species. Between pH 8 and 10, the 1 1 -1 species is the dominant one, only been replaced by the 1 1 -2 species at pH > 10.3. The deprotonation constant of the 1 1 -1 species ($\log\beta_{11\text{-}1} - \log\beta_{11\text{-}2} = 12.69 - 2.49 = 10.2$) matches closely the 10.24 value for the deprotonation of the ε-NH_2 side group of

the Lys residue (Table 1), suggesting the last deprotonation process for Cu(II)- complex involves the removal of the proton from an unbounded ε-NH_3^+ (Lys) group.

The stability constants for the Cu(II)-Asp-Thr(Ac)-His-Lys complexes are generally higher as compared to other similar Cu(II) complexes of tetra or tri-peptide containing His in the third position, like Lys-Gly-His-Lys [51], Gly-Gly-His [52], and Asp-Ala-His-N-methylamide [23].

3.4. UV-Visible studies of the Cu(II)-peptide Complex System

3.4.1 Cu(II)-His-Lys

The UV-Visible spectra of a solution containing a 1:1 mixture of Cu(II) and His-Lys at a concentration of 6.6 mM is shown as a function of pH in Fig.7. In order to propose structures for the possible complexes formed from Cu(II) and His-Lys mixture, Billo's [44] empirical equation (1) can be used, based on the observed λ_{max} value in visible absorption spectrophotometry,.

$$\lambda_{max} = 10^3 / \sum_i n_i \nu_i \qquad (1)$$

where n_i is the number of the donor atom (i) in the complex ($\sum n = 4$). The coefficient ν_i (wavenumbers in μm^{-1}) is the ligand-field contributions of the donor atom (i). The coefficients (ν) for various donor groups have been calculated by different investigators as shown in Table 5.

According to the equation 1, the replacement of water molecules by oxygen or nitrogen donors in Cu(II) shifts λ_{max} towards lower values (blue shift), the effect of the nitrogen donors being more marked. In Fig.7, there is a progressive blue shift as pH increases, an indication of the involvement of oxygen and better still nitrogen atoms in complex formation. The observed maximum can be used together with other information about the coordinating groups that are available to bind to a Cu(II) center to construct a ligand set that would produce the observed spectral maximum [44]. Also, based on the selected set of coordinated ligands, it is possible to make intelligent guess as to the stoichiometry of the complex formed as illustrated further in the discussion.

All possible logical permutations (~ 120) of the donor groups listed in Table 5 that will sum up to four (assuming square planar complexes) were entered and calculated in an Excel® spreadsheet and matched with the observed λ_{max} at different pH values to choose the likely coordination set of donor atoms. Table 6 summarizes the possible donor atoms for the various complex species at different pH values for equimolar solution of Cu(II) and His-Lys, based on closeness of the experimental λ_{max} to the calculated values using equation 1 and the data from Table 5. There is generally, a good agreement between the observed and all the calculated λ_{max} for the various species. The spectral data obtained for the major species formed at pH 3.3 (which by the potentiometry study is a 1 1 2 (Fig 4a)), suggests an N donor and O (carboxlyate) coordination to the Cu(II). The three calculated λ_{max} using the different coefficients in Table 5 for a coordination mode involving 1 NH_2, 1COO^-, and 2 H_2O agrees excellently with observed λ_{max} of 715 nm at pH 3.3. A calculated λ_{max} for the coordination mode involving 1 peptide N, 1carboxylate and 2 H_2O, i.e. 700, 692, 700 nm using the respective coefficients from [44], [45] and [46] (Table 5) is also close to the observed λ_{max}. Despite the closeness of the calculated values to the observed λ_{max}, this coordination mode does not seem likely since metal ion induced deprotonation and coordination of amide nitrogen are well known to occur only after the initial coordination of an amino group [39] or in the case of His-peptides, after the initial coordination of the imidazole N [40] but *not* carboxylate groups nor, of course, H_2O. In the presence of both terminal amino and α-His residue, the N-3 of the imidazole ring of the His residue has been reported to be the primary anchoring site for Cu(II)-coordination [40]. The calculated λ_{max} for coordination mode involving 1 N (imidazole), 1COO^-, and 2 H_2O, however, seem slightly higher than the observed, possibly because of contributions of NH_2-Cu coordination from other species. The observed λ_{max} of 673 nm for the equimolar solution of Cu and His-Lys at pH ~ 6 agrees well with all three calculated λ_{max} for the coordination mode involving 1 NH_2, 1 imidazole N, and 2 H_2O (Table 6). Based solely on the spectral data, it is reasonable to suggest that a complex of the structure as shown in Fig. 8a is formed at pH around 6. However, from the pH potentiometric study above, the major species formed around pH 6 is the binuclear 2 2 1 complex (Fig 4A). Structures similar to that found for L-carnosine in the solid state [53], where the two metal centers of the two monomeric complexes are bridged by the N-3 (Im) donors and each metal center possessing an (NH_2, N (imidazole), N (amide), and COO^-) coordination are normally

assumed for binuclear complexes of di-peptides, X-His (X = amino acid) in aqueous solutions. In the case of His-Gly for example, the assumption is reasonable, since the λ_{max} for the Cu(II)-peptide solution at the pH that corresponds to the formation of the reported binuclear complex (2 2 -2) is 600 nm [54], in accordance with the coordination environment (3N and 1O) around each Cu(II) ion as similarly observed in the structure of L-carnosine. In this present study, however, the λ_{max} obtained for the Cu(II)-His-Lys equimolar solutions at the pH where the binuclear complexes 2 2 1 and 2 2 0 are formed do not suggest such coordination mode. A plausible structure for the species 2 2 1 detected in this study is shown in Fig. 8b. It is worthy of mention that hydroxyl-bridged dimeric species for Cu(II) complexes of some peptides (e.g. Gly-Gly) are known [55]. The deprotonation of 2 2 1 gives rise to the 2 2 0 binuclear species, which by the spectral data, may be as a result of the involvement of the amide N in the coordination, or may also be due to the deprotonation of one of the coordinated water molecules.

The binding of the imidazole N and the α-NH_2 groups in the 2 2 1 species indeed serves as an anchor, that facilitates the deprotonation and the coordination of Cu(II) to the deprotonated peptide N (N^-) as pH increases to about 8.4. A new complex species $Cu(HL)H_{-1}$ (corresponding to the 1 1 0 species in Fig 4A) is formed, where H_{-1} indicates dissociation of a proton from the peptide moiety and Cu(II) is coordinated to imidazole N, a deprotonated peptide nitrogen (N^-), 1 α-NH_2 and 1 COO^- (Fig.8c). The observed λ_{max} = 542 nm at pH 10.3 (Fig. 7) agrees closely with the calculated value of ~ 541 nm for 2 amino N, 1 amide N and 1 imidazole N (Table 6). The coordination mode suggest the involvement of the ε-NH_2 group in the coordination to the Cu(II). The deprotonation constant of the 1 1 0 species ($\log\beta_{110} - \log\beta_{11-1} = 12.79 - 3.29 = 9.5$), which corresponds to the deprotonation of the ε-NH_2 side group of the Lys residue, is significantly reduced to confirm the involvement of the ε-NH_2 side group in the coordination to the Cu(II) ion as suggested by the spectral data. the last deprotonation process for Cu(II)- complex involves the removal of the proton from an unbounded ε-NH_3^+ (Lys) group. The likely structure of the complex formed is shown in Figure 7d.

Table 7 summarizes the λ_{max} and the possible donor atoms for the various complex species at different pH values for mixture of 2:1 His-Lys and Cu(II). equimolar solution of Cu(II) and His-Lys, based on closeness of the experimental λ_{max} to the calculated values using equation 1 and the data from Table 5. There is generally, a good agreement between the

observed and the calculated λ_{max} for the various species. As stated earlier, the presence of excess ligand prevents the formation of the binuclear complex. At the pH region where the binuclear complexes are expected, the bis-coordinated complex
($Cu(HL)_2$) with a coordination mode of (2N(imidazole) and 2 COO^-) are formed. At higher pH however, similar monomeric complexes as found for the equimolar solutions are formed.

3.4.2 Thr(Ac)-His-Lys

The UV-Visible spectra of a 1:1 mixture of Cu(II) and Thr(Ac)-His-Lys (12.0 mM) in the pH range 2.7-11.9 is shown in Fig. 9. Unlike the Cu(II) and (His-Lys) system (*vide supra*), as the pH increases from 4.3 to 8.5 the observed λ_{max} stays practically the same, i.e. 600 ± 5nm. This value is in excellent agreement with the predicted value of 599 nm [44,45] and 595 nm [46] of 1 amino N, 1 amide N, 1 imidazole N and 1 oxygen atom of either COOH or H_2O. This suggests a simultaneous binding of these donor atoms to Cu(II) for Thr(Ac)-His-Lys in contrast to the successive deprotonation and subsequent binding of each of the ligand for His-Lys as pH increases from ~ 4 to 8. One major species, $Cu(HL)H_{-1}$ (1 1 0) seem to be formed between pH 4.3 to 8.5 (Figure 10a) with the maximum absorptivity (ε_{max} = 77 dm^3 mol^{-1} cm^{-1}) occurring at pH ~8.5 .

The above observation is in accord with the difference in the acid-base speciation of the two peptides (not shown). Unlike His-Lys, the pK_a values for Thr(Ac)-His-Lys signifying the protonation of the imidazolium group and the α-ammonium groups are closer as compared to that for His-Lys [30]. Thus, the HL species in Thr(Ac)-His-Lys are formed in significant amounts over a wide pH range of ~ 5 to 12 and dominate all the other species over still a wide pH range of ~ 7 to 11. The dominance of the H_2L in
His-Lys for the pH range ~5.5 to 7, however limits the pH range where HL is the dominant. Thus unlike Thr(Ac)-His-Lys, as pH is increased from ~ 4 to 8, different species dominant, and these in preference over the minor ones form different and more Cu(II) complexes with the various His-Lys species.

Beyond pH 8.5 however, there is deprotonation of both the ε-NH_3^+ proton and the other NH to form a coordination mode involving 2 amide N atoms, 1 amino N,

1 imidazole N (Fig. 10b) whose predicted λ_{max} = 540 nm [44] is in agreement with the observed λ_{max} 541 nm at pH 11.9. This type of bonding has been observed in an X-ray study of Cu(II)-Gly-Gly-His-N-methylamide [48]).

The positions of the absorption bands (λ_{max}) of the Cu(II)-peptide complexes in solutions of peptide to metal ratio 2 at different pH values are shown in Table 8. In solutions of excess of the tripeptide over the Cu(II) ion, the bis-complex $Cu(HL)_2$ with a 3N, O coordination environment around the Cu(II) dominants in the entire pH range from 2 to ~ 8. The coordination mode, suggests that each of the two peptide, coordinates via the imidazole N and one of either COO^- or the α-NH_2 N group. As the pH increases, the amide protons are also deprotonated in succession, leading to its involvement in the formation of the other species at higher pH as depicted in Table 8.

3.4.3 Asp-Thr(Ac)-His-Lys

The UV-Visible spectra of 1:1 Cu-(Asp-Thr(Ac)-His-Lys) of concentration of 3.0 mM in the pH range 2.80 to 12.20 is shown in Fig. 11. As shown in Table 9, there is generally, a good agreement between the observed and all the calculated λ_{max} for the various species using equation 1. The almost featureless spectra obtained between pH 2.8 and pH 4.0 indicates no significant coordination of Asp-Thr(Ac)-His-Lys to Cu(II). Between pH 6.5 to 9 broad peaks at λ_{max} 576 ± 8 nm are observed for bright blue aqueous solutions which are due to coordination of O and /or N-donor groups to Cu(II). The average predicted λ_{max} for the visible absorption spectra using Billo's equation above and coefficients obtained from the three different sources [44,45,46] when the coordinating groups are an amino, a peptide N^-, a carboxylate and a N(3)-imidazole nitrogen is about 581 nm as also observed experimentally (see Table 9). The species so formed reaches its maximum absorptivity (ε = 86 $dm^3\ mol^{-1}cm^{-1}$) at pH ~ 9. There is then a gradual blue shift as the pH of the solution is increased above 9, an indication of the involvement of more nitrogen atoms in the coordination to form another species (purple in color). The formation of this species seems to be complete (maximal absorptivity) when the pH = 11.2. The λ_{max} of the species is 541 nm and the absorptivity is 112 $dm^3\ mol^{-1}\ cm^{-1}$. This λ_{max}

value agrees very well with a calculated one of 539 nm (Table 9) for the coordinating groups of two dissociated peptide, 1 amino and N(3)-imidazole nitrogens.

3.5 Cu(II) complexation of Asp-Thr(Ac)-His-Lys in solutions of pH 7 by ^{1}H NMR

3.5.1 Paramagnetic broadening

Fig.12 (b to g) shows the ^{1}H NMR spectra for the amide protons, ammonium protons and aromatic proton region obtained from a phosphate buffer solution at pH 7.0 of a 9.6 mM Asp-Thr(Ac)-His-Lys aqueous solution as different amounts of Cu(II) are added. Fig. 12a shows the ^{1}H NMR spectra of these peaks in water adjusted to pH~3, and demonstrates the substantial shift in the chemical shifts of some of the resonances as pH changes from ~ 3 to 7.

The disappearance of the imidazole C-2 signal, at a chemical shift of ~ 8.1 ppm, and of the His β-CH_2 protons, at a chemical shift of ~ 3.2 ppm, when about a fifth of an equivalent of Cu(II) is added (Fig. 12e) is due to the paramagnetic broadening effect. Although there is substantial reduction of the peak intensity of the Im C5-H peak at ~7.12 ppm under these conditions, suggesting some proximity to the paramagnetic Cu(II), this signal and the imidazole N1-H were both recognizable even at a Cu:peptide ratio of 1:1. These observations support the idea that the N3 and not the N1 is the binding site for the imidazole group in this complex.

Furthermore, the spectra before adding any Cu(II) in both non-buffered aqueous solution (Fig.12a) and in buffer 7 solution (Fig. 12b), and peak integrations, indicate the presence of another resonance (Im N3-H) overlapping with the C5-Im at the chemical shift (~7.12). The Im N3 peak broadens out immediately on adding the initial amount of Cu(II) and also shifts downfield to make it barely recognizable, being buried under C5-Im (see Fig. 12c). This further supports the idea of Cu(II) binding initially to Im N3.

At Cu(II):peptide ratio > 1:1, the overlapping signals of Thr- and His- amide NH completely disappear. This could either be due to the removal of these protons, or as a result of paramagnetic broadening, or both. In either case, this indicates binding of Cu(II) to both amide Ns. By contrast, the Lys amide NH signal at a chemical shift ~8.5 was still visible at ~ 1:1 stoichiometry (Fig. 12g) and even at a two-fold excess of Cu(II) over peptide it was still present

(not shown). Consistent with this, the well resolved doublet due to the γ-Thr CH_3 protons also broadens out gradually upon the initial addition of Cu(II), to result in a broad singlet when about a third of an equivalent of Cu(II) has been added.

Fig 13. shows the changes in the chemical shift of the C-2 imidazole proton and β-His CH_2 as Cu(II) is added. The chemical shift changes as a function of the Cu/peptide ratio are both linear with correlation coefficients of 1.00 for the C-2 imidazole and 0.98 for the β-His CH_2 protons. Although the C-2 imidazole proton is closer to the N-3(Im) than the β-His CH_2 protons, the changes in the chemical shifts are comparable. This suggests the involvement of another site in the proximity of the β-His CH_2 protons (i.e. the His-amide NH). This is confirmed by the fact that the His-amide NH experiences substantial chemical shift changes, even than the C-5 imidazole proton (Fig. 14).

As shown in Fig. 15, the chemical shift of the CH_3CO-Thr protons are more affected upon adding Cu(II) than the γ-CH_3-Thr protons. The observed changes cannot plausibly be attributed Thr peptide N coordination, since these protons are several bonds away. A through space interaction with a binding site in close proximity i.e. COOH (Asp) is implied. Note from Section 3.1 that the acid-base behavior of the free peptide indicates an orientation where the CH_3CO-Thr protons are in close proximity to the COOH(Asp).

It is noteworthy that the above arguments do not preclude the binding of Cu(II) also to the Thr amide nitrogen.

The delayed initial rise for both curves a and b in Fig. 15 seem to suggest that some other activity than the Cu(II) complexation occurs at low Cu:peptide ratio. The abrupt tailing off for curve b at a Cu:peptide ratio of 0.5 (i.e. peptide:Cu ratio of 2:1) seem to suggest a possible formation of a peptide-Cu complex of stoichiometry 2:1, although such species were not detected by the potentiometric study.

Since the δ-CH_2 protons of the peptide display larger chemical shift changes as Cu(II) is added than the ε-CH_2 protons, it appears that the ε-NH_2 of Lys is not involved in Cu(II) complexation at pH 7. An earlier report which observed similar changes in chemical shift of Lys protons as Cu(II) was added to both BSA and HSA, attributed such changes to a conformation where the Lys ring lie over the imidazole ring of His [16].

3.6 Proposed Structure for Cu(II)-(Asp-Thr(Ac)-His-Lys) at pH 7

Despite the similarities in the amino acid sequence, HSA models cannot effectively model the activity of BSA, since "minor" changes such as replacing Ala with Thr can lead to differences in their coordination ability. For instance, the pK_as for the β-carboxylate, the imidazole and the amino groups were measured to be 2.98, 6.66 and 7.73 for the HSA model Asp-Ala-His-N-methylamide [23] as compared to 4.91, 6.55, and 8.74 for the BSA model Asp-Thr(Ac)-His-Lys in this study. The difference in the pK_a values for the β-COO^- and α-amino protons are very substantial and would therefore result in a significant difference in their species distribution with respect to pH. The complex species that would be formed would therefore also be expected to differ significantly.

The ^{1}H-NMR data clearly indicate the involvement in Cu(II) binding of the N-3 His nitrogen, the amide nitrogens of His and Thr and another site closer to the Thr-side chain (the β-carboxylate and/or the α-amino nitrogen). The β-carboxylate seems to be the more likely site, since the CH_3CO-Thr protons that sense the Cu(II) coordination at this site, sense the β-carboxylate and not the α-amino when the free peptide is deprotonated. The ε-amino N of Lys is clearly not involved.

The visible absorption data of Asp-Thr(Ac)-His-Lys and Cu(II) mixtures at pH 7.1 display a broad absorption maximum centered at 576 nm, whose closest predicted (averaged) λ_{max} based on the three coefficients in Table 5 are respectively 581 and 569 nm for the two possible coordination modes {N^-, $1NH_2$, $1N_{im}$, $1COO^-$} and {$2N^-$, $1N_{im}$, $1COO^-$} (see Table 9 for details). Since the involvement of the imidazolium, carboxylate, and the amide nitrogens of His and Thr are clearly indicated by both the NMR and visible spectroscopic studies at pH ~ 7, the latter coordination is implied at that pH value. Nevertheless, the former coordination also needs further consideration, since the NMR study, in fact, did not rule out the possibility of the involvement of the α-NH_2, but rather suggested its remote involvement. The observed λ_{max} (581 nm) at pH 6.5 is in excellent agreement with a predicted value of 580 nm for {1 N^-, 1 NH_2, 1 N_{im}, $1COO^-$} coordination based on ν values of [46], whilst the value observed at pH 9 (569 nm) is in excellent agreement with a predicted value of 567 nm for {2 N^-, 1 N_{im}, $1COO^-$} coordination also based on ν values of [46]. This suggests

that the visible absorption spectra observed at approximately pH 7 is due to an equilibrium mixture of these two species.

Since pK_{a3} of Asp-Thr(Ac)-His-Lys is 8.7 (i.e. deprotonation at N-terminal amino group), H_2L is the dominant species in the pH range of ~ 6.5 to 9. As pH is increased through the vicinity of pK_{a3}, competition by protons for the α-amino coordinating site becomes less effective making it easier for Cu(II) to bind this site. At pH > 6.5, a simultaneous binding of Cu(II) to the imidazole N-3 nitrogen, carboxylate, a deprotonated peptide N, and the α-amino group to form a $Cu(HLH_{-1})$ complex species is therefore possible, as shown in Fig. 17a. The stoichiometry of this species also agrees with the 1 1 0 species identified by the potentiometric studies as the dominant species for the Cu(II)-tetrapeptide complex system (Fig.6). Further increase in pH value towards 9 can cause Cu(II)-induced deprotonation of the second amide NH bond [22], that is Thr amide-NH, and lead to the formation of the $Cu(HLH_{-2})$ complex species, as shown in

Fig. 15b, which concurs with the observed speciation pattern shown in Fig. 6. Both these species, $Cu(HLH_{-1})$ and $Cu(HLH_{-2})$, involve the coordination of the carboxylate group to Cu(II), in agreement with previous ^{13}C NMR findings by Lussac and Sarkar [15] for the HSA model , Cu-(Asp-Gly-His-N-methylamide) complex system between pH 4 and 6.

Between pH values 9 and 11 where HL and L are the dominant species for the free peptide (diagram not shown) the weaker coordination by the carboxylate group is replaced with the α-amino group to form the $Cu(LH_{-2})$ species, with the 4N coordination involving the α-amino group, 2 peptide N- and the Im N-3, as shown in Fig. 17c). As explained earlier the deprotonation of the ε-NH_3^+ (Lys) proton from the $Cu(HL)H_{-1}$ occurs at the same pH vicinity as in the free peptide, so by the pH ~ 10.2, it would be deprotonated to form the $Cu(LH_{-2})$ complex, as shown in Figure 17c. This results in a visible absorption band with λ_{max} = 541 nm, consistent with theoretical prediction of

539 nm based on Billo's equation [44] for such coordination (Table 9). The studies of the Cu-(Asp-Gly-His-N-methylamide) complex system as a model of HSA suggested the same coordination environment as the $Cu(LH_{-2})$ species (Fig. 17) but at a lower pH, between 6 to 10.

3.7 Binding Strength of Cu-(AspThr(Ac)-His-Lys)

The NMR chemical shift of the imidazole C5-H proton of Asp-Thr(Ac)-His-Lys (9.6 mM) in a buffer solution of 7 was measured as a function of [Cu(II)]. See Fig. 18. The equilibria involved in the (Asp-Thr(Ac)-His-Lys)-Cu(II) aqueous system at pH 7 can be expressed as follows:

$$H_3L \;\rightleftharpoons\; H_2L \;\rightleftharpoons\; HL \;\overset{K_{fl}}{\rightleftharpoons}\; (HLH_{-X})Cu$$

(K_{a1} between H_3L and H_2L; K_{a2} between H_2L and HL)

As discussed earlier, the two main Cu(II)- complexed species of Asp-Thr(Ac)-His-Lys Cu-(HLH_{-1}) and Cu-(HLH_{-2}) both involve the HL species of the peptide; since the difference between the two is the loss of a proton, with a value of a pKa connecting the two, at any given pH the ratio of the two is constant. Hence for simplicity these two species are treated as one composite species, Cu-(HLH_{-x}). Only one averaged chemical shift, δ_{obs} , is actually observed for each resonance, indicating that exchange between free and Cu(II)-complexed Asp-Thr(Ac)-His-Lys species is fast on the NMR timescale. The observed chemical shift in each case is therefore the average of those of the individual species, weighted by their relative amounts:

$$\delta_{obs} = \delta_{H3L}\alpha_{H3L} + \delta_{H2L}\alpha_{H2L} + \delta_{HL}\alpha_{HL} + \delta_{CuHLHx}\alpha_{Cu(HLHx)}$$

where δ_x refers to the chemical shift of a given species x, and α_x its mole fraction. α values can be calculated from the acid dissociation constants, formation constants and the pH values of the solutions. The pH in this case is constant at 7.0. The calculation is done based on mass action equation for the equilibrium process above and the mass balance equations for Cu and the ligand

L; from these equations the quadratic below can be obtained and solved for [Cu] at equilibrium for each data point:

$$[Cu]^2.K_f + [Cu]\{1/\alpha_{HL} + K_f(C_L - C_{Cu})\} - C_{cu}/\alpha_{HL} = 0$$

Since the chemical shifts and the acid dissociation constants of free Asp-Thr(Ac)-His-Lys were determined in Section 3.1, the only unknown parameters in the two equations above are the formation constant and the chemical shifts of the $CuHLH_{-x}$ complex species. Non-linear least squares regression analysis as described in Section 3.1 can be used to fit the predicted chemical shift to the observed chemical shift in an attempt to extract the chemical shifts and the formation constant of the Cu(HL) complex species. The resultant fit of predicted to experimental values are shown in Fig. 18. The predicted fit was found not to depend on the value of K_f. The linearity of the relationship (Fig. 18) suggests that the equilibrium lay well to right for the complexation of Cu(II) to the tetrapeptide at pH 7. It should be noted that, if the binding is sufficiently strong, then the mole fraction of complexed formed (i.e.αCuHL) is simply proportional to the amount of Cu added.

An alternative approach to estimating strength of binding is to set up a competition for Cu(II) by the peptide and another ligand of established formation constant. Such competition was carried out between Asp-Thr(Ac)-His-Lys and Lysine,
by observing the chemical shift changes of Im C5-H as increasing amounts of Cu(II) were added to the peptide, in the presence and absence of 1.25 mole equivalents of Lys (Fig. 19). In the absence of any interaction between the Lys and the tetrapeptide, the chemical shift of the Im C5-H proton of the peptide should be the same in the presence or absence of the Lys when no Cu(II) has been added. This is not the case in Fig. 19, suggesting some interaction between the Lys and the peptide, perhaps hydrogen-bonding interaction.

As expected, the chemical shift in both the presence and absence of Lys increases monotonically. The presence of Lys decreases the slope from 0.115 to 0.085, indicating that Lys competes with Asp-Thr(Ac)-His-Lys for Cu(II) and that the latter exhibits 3.5 (i.e. (0.085*1.25)/(0.115-0.085) fold stronger binding to Cu(II) than does Lys. (This is only true assuming no ternary complexation between Lys, the peptide and Cu(II) occurs). Cu(II) complexation to Lys has been well studied over a pH range of 2-13 [28,56,33]. The dominant

species at neutral pH is CuL_2H_2 with a log β = 35.16 formation constant at 25° C and ionic strength of 0.2 M [33]. The formation constant of Cu-(LHx) complex species of the Cu-(Asp-Thr(Ac)-His-Lys) system at pH 7.0 is therefore log β =35.70
(i.e 35.16 + log 3.54). This value concurs with the log β_{113} = 35.55 discussed earlier and obtained by potentiometry.

4. CONCLUSIONS

As part of the broad objective of investigating interactions between Cu(II), TMs, and BSA, the acid-base behavior of the BSA model peptides His-Lys, Thr(Ac)-His-Lys, Asp-Thr(Ac)-His-Lys was studied by ^{1}H NMR ,and Cu(II) complexation by both ^{1}H NMR and visible spectroscopy. Despite the paramagnetic nature of Cu(II) ions, solution ^{1}H NMR studies proved very insightful in elucidating the binding sites and the relative strengths of Cu (II) binding to these peptides. Complemented by some visible absorption data, this study proposed structures for Cu(II)-HisLys, Cu(II)-Thr(Ac)-His-Lys and Cu(II)-Asp-Thr(Ac)-His-Lys systems in solution at different pH values. Potentiometry has also been used to obtain reliable and formation constants for the various species for the Cu(II)-peptides complex systems.

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Abbreviations

Boc	tert-butyloxycarbonyl
Bzl	benzyl
Z	benzyloxycarbonyl
Ac	acetyl
HOBt	1-hydroxybenzotriazole
DNP	dinitophenyl
TFA	trifluoroacetic
EDC	1-(3-Dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride
BSA	Bovine serum albumin
HSA	Human serum albumin
DSS	sodium 2,2,-dimethyl-2-silapentane-5-sulfonate
TMS	tetramethylsilane
Im	imidazole
NMR	nuclear magnetic resonance

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COPPER DIETARY SUPPLEMENTS AND THEIR BIOAVAILABILITY IN RUMINANTS AND OTHER ANIMALS

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INTRODUCTION

Archeological records suggest that copper (Cu) has played a significant role in civilization since its early use in hammered artifacts of the stone age (McDowell 1992). Its therapeutic role has also been known since Hippocrates prescribed copper compounds for pulmonary and other diseases (Mason 1979). The use of copper compounds in the treatment of diseases reached its peak in the 19th century and subsequently declined when the treatments were not successful (Turnlund 1999). The essentiality for ruminants was first established in 1931 when Cu deficiency in grazing cattle was demonstrated in Florida (McDowell 1992). Copper is now well known to form an integral component of many enzymes (Sarkar 1999), and therefore an essential element in ruminant nutrition. Its deficiency is often a problem in cattle (Ward et al. 1996) and in other ruminants. Copper deficiency in cattle can lead to significant production losses. Acute deficiency is often characterized by depraved appetite, diarrhea, anemia, infertility, rough haircoat, faded hair color, and severe growth retardation (Suttle 1991, Mason 1978).

The pathologies associated with Cu deficiency can be reversed using dietary copper supplementations (O'Dell 1976, MacDonald et al. 1988, Church 1988). Ruminant dietary deficiency in Cu have usually been corrected in the past by administration of "inorganic mineral supplement," i.e., simple Cu salts (Church 1988). The bioavailabilities of Cu in these substances, however, have been shown in some cases to be low (Ashmead et al. 1986, Howell and Gawthorne 1987). To circumvent this problem of Cu availability, "organic chelated minerals" i.e., Cu in the form of a complex bound to a ligand or ligands, therefore have been proposed as a better alternative (Spears 1993). These are thought to be more bioavailable and potentially less toxic (DeBonis and Nockels 1992, Kincaid et al. 1986). It has been speculated that this might be due to their persistence in the gastrointestinal (GI) tract of animals (Nelson 1988).

Compounds such as Cu-lysine, Cu-Glycinate, Cu-CaEDTA, Cu-gluconate, Cu-oxyquin (diethylamine copper oxyquinoline sulphonate) and Cu-methionate have therefore been used either orally or parenterally (Judson et al. 1982, Allen 1987, Smart et al. 1992, Rombach et al. 2003). However, there are conflicting reports regarding their advantage over simple "inorganic" forms of copper supplementation for ruminants (Luo et al. 1996), especially in the presence of dietary Mo. The major shortcoming of "chelated minerals" is their higher cost. In an earlier paper (Attaelmannan and Reid 1996), we compared Cu(II)-lysine to Cu(II) Chloride (in the

absence of any Mo compound). The results of the Cu speciation in bovine saliva, the first biofluid encountered in gastrointestinal tract, indicate virtually identical speciation in the two cases.

Most naturally occurring Cu deficiencies in livestock are secondary rather than primary (Smart et al. 1992). Secondary deficiencies are those that refer to dietary factors in the plant or soil that interfere with absorption and availability of Cu (Underwood 1977) rather than a low dietary Cu concentration (primary). Bovine Cu deficiency is common in regions of high pasture Molybdenum (Mo) concentrations (Suttle 1991) and cattle appear to be more susceptible to Mo-induced Cu deficiency than sheep (Gooneratne et al. 1989). Thiomolybdates (TMs) formation in the rumen is now well established. These TMs react with Cu(II) in the gastrointestinal (GI) tract rendering it bio-unavailable (Suttle 1991, Sarkar and Mishra 1984). These TMs, particularly the tetrathiomolybdate, TM4 (Gooneratne et al. 1989, Sarkar and Mishra 1984, Mills et al. 1981) are probably the most effective secondary cause of copper deficiency. Would chelated Cu supplements escape the influence of TMs and permit the absorption of Cu intake?

In the light of the information obtained from solution chemistry studies involving copper speciation under mimicking conditions of some biofluids in ruminants (Attaelmannan and Reid 1996, Reid and Attaelmannan 1998) as well as the interaction between Cu and TMs (in the presence and absence of some ligands) (Quagraine and Reid 2001, Quagraine 2002, Clarke and Laurie 1982, Laurie et al. 1986, Clarke et al. 1987, Wood and Mason 1987), we wish to examine the role of Cu supplements in bovine and other rumen diets as studied in the field of animal science. The performance of both the inorganic and the Cu-chelated supplements, in the presence and absence of Mo or TMs will be assessed with especially the cost involved in their administration in mind. Despite the fact that the focus of this review is on ruminants, extension have also been made to some studies involving monogastric animals such as pigs, rats and chickens whenever found necessary to connote the generality of the issue under discussion.

COPPER SUPPLEMENTATION

As stated earlier, many of the symptoms associated with Cu deficiency can be reversed upon supplementing diets with Cu. Trace mineral supplementation is recommended for livestock

to ensure that the animal's mineral requirements are adequately met. The objective of any method of supplementation must be to satisfy an animal's requirements for Cu, efficiently and at a minimum cost (Allen 1987). Once these requirements are met, the producer's acceptance of the supplement often depends also on the ease by which the supplement can be used (Smart et al. 1992).

Copper Requirements

Cattle copper status is difficult to assess as it depends on a number of interdependent factors, including dietary copper, nutrient interactions, age and breed, season of the year and climatic conditions (Smart et al. 1992). The National Research Council (NRC 1996) establishes estimates of copper requirements.

For meaningful recommendations of Cu requirements it is necessary to make reference to molybdenum and sulfur concentrations in the feed, pasture or soil. Nonetheless, for cattle a dietary level of 10 mg Cu Kg^{-1} of dry matter (DM) is recommended, with a range of 4 to 15 and a maximum of 100 mg Cu Kg^{-1} (NRC 1996). With very low levels of Mo (below 1.5 ppm), 4 ppm (mg Cu Kg^{-1}) has been found to be adequate. Other important factors such as interactions with other minerals should also be taken into consideration. Cu:Mo ratio in silage has been found to be a very important factor in determining the number of services by artificial insemination required by dairy cattle in the lower Fraser valley of British Columbia in Canada (Peterson and Waldern 1977). Miltimore and Mason (1971), reported that a Cu: Mo ratio of 2:1 appeared to be critical in preventing conditioned Cu deficiency in cattle in this province. In that same province, 19 % of all ruminant feeds were of ratios less than 2:1. Copper deficiency has been reported not only in that province but also in most Canadian provinces (Smart et al. 1992, Goorneratne et al 1989). In Argentina, hypocuprosis in animals have been reported (Bingley and Carrillo 1996) when the Cu:Mo ratio of forages in the presence of adequate S is less than 2.8:1. Alloway (1973) has also suggested a minimum Cu:Mo ratio of 4:1 to met the Cu requirements. In other cases a Cu:Mo ratio of 3:1 has been regarded as a minimum, 4.3:1 as adequate and 6:1 to 10:1 as ideal (Puls 1988). The stoichiometry for Cu(II) interactions with TM3 and TM4 (the more potent Cu (II) antagonists) is 1:1 (Quagraine and Reid, 2001). However under physiological pH, higher Cu(II):Mo ratio (1.5:1 (Laurie et al. 1986) is required to drive the reaction to completion. Dietary

Cu:Mo ratios should exceed 1 (more correctly probably 1.5) to enable the effective absorption of Cu(II) (Quagraine and Reid, 2001). The relatively higher dietary Cu:Mo ratios required to prevent Cu deficiency in the various feed studies may be due to allowance created for the contribution of endogenous losses to fecal Cu excretion (Suttle 1987) and the interaction of Cu(II) with some substances in the rumen. As well, the variable ratios quoted for these studies may reflect the different dietary levels of these substances in the various localities. Examples of substances that have been reported to impede Cu absorption apart from the well known TMs and S^{2-} are; Histidine, phytate, ascorbate, cysteine, D-penicillamine, fiber, carbohydrates, Zn, Fe, Se, Pb, Ni, Cd, Hg, Ag (Cousin 1985, Underwood 1977, Gooneratne 1989, Hunt and Groff 1990, Gawthorne 1987, Turnlund, 1999, Aoyagi and Baker 1994). Furthermore, how Cu supplements are administered may also influence the requirements, since for instance, dietary Cu in most species is poorly absorbed, only about 5% is available (Mason et al. 1988).

As stated by Smart et al. (1992), before the type and amount of Cu supplementation is chosen, the nutritionist must not only know the Cu concentration in the feed, but also the concentration of all dietary elements which influence dietary Cu availability.

Types of Cu supplements

The types of supplements range from simple inorganic supplements to forms in which the metal is bound to amino acids, large organic molecules, or proteins. These last three groups have been classified together in the nutritional field as "chelated"–Cu supplement and have been assigned some uniqueness over the inorganic supplements. Special attention is therefore given to this type of Cu supplementation in this review paper at a latter section. The traditional method of supplementation is however through the use of inorganic metal supplements (Wittenberg 1993). The most common form of Cu supplementation in Canada involves the use of $CuSO_4$ (Wittenberg and Biola 1988). It is considered as the standard form, against which other inorganic and "chelated" supplements are compared. The relative uptake of ^{64}Cu from several inorganic compounds by beef cattle indicated by the presence of ^{64}Cu in the blood is the order: $CuCO_3$ > $Cu(NO_3)_2$ > $CuSO_4$> $CuCl_2$> Cu_2O > CuO (powder) >CuO (needlees > Cu(wire) (Underwood 1977). On the contrary, supplemental $CuSO_4$ has been found to show more biological availability of Cu than $CuCO_3$ (McDowell 1992).

Different Modes of administering Cu supplements

There are a number of ways of administering supplemental copper to the ruminant. Smart et. al 1992 and Allen 1987 in their separate reviews and McDowell 1992 in his book gave details with regards to how the different modes of Cu supplementation are administered and hence such details have been omitted in this current review to avoid unnecessary duplication. In this section, the individual modes of Cu supplementation will be mentioned briefly (details are provided only when deemed necessary) with the view of comparing their effectiveness and cost.

When ruminants are pen-fed or in a dry lot feeding, supplementary Cu is best supplied by incorporation of the element into a concentrate mixture. This method is not, however, recommended for grazing ruminants, as it is extremely expensive (McDowell 1992). Many Cu compounds have been evaluated as soil and foliar fertilizers (Gartrell 1981), otherwise called it top dressing. Unfortunately, responses are not consistent and the cost of repeated top dressing is prohibitive (Allen 1987). Cu can also be supplemented in drinking water (Farmer et al. 1982), however this method (Smith and Moon 1976) was found to be less satisfactory than other methods of supplementation. Salt licks are also well known but the intake by ruminants, which are grazing will certainly be irregular and difficult to maintain the availability of the Cu mineral. Some may take too little or take too much of the lick and die from Cu poisoning (Allen 1987). Other techniques involve dosing or drenching animals with Cu compounds or injection of organic complexes (McDowell 1992). Subcutaneous or intramuscular injection of some forms of Cu, those that are slowly absorbed, is a satisfactory means of treating Cu deficiency. For severe cases of molybdenum induced Cu deficiency, injection of Cu compounds is the recommended procedure, as it circumvents the gut – the main site of copper/molybdenum interactions. More than 75% of a single dose may reach the liver for storage compared with less than 10% of a single oral dose (Allen 1987). The number of doses required depends on the severity of the Cu deficiency and the type of Cu complex administered. For effectiveness, its administration is recommended every three months (Smart 1992) in cattle fed with low Cu diets. Injection of Cu as Cu-glycinate at 6-wk intervals is effective in maintaining Cu in plasma at normal concentrations (Klein et al. 1985). Its main disadvantages are the fact that in most cases the injection causes reactions at the site leading to swellings and sometimes progresses to form a

sterile abscess (Allen 1987). The use of injectable Cu preparations for the prevention and treatment of Cu deficiency have resulted in deaths of; sheep and Ewes given a subcutaneous single dose of 50 mg Cu-CaEDTA, cattle given a subcutaneous single dose of 72-144 mg Cu(II)-bis-8-hydroxyquinoline and sheep given an intravenous single dose of 50 mg $CuSO_4.5H_2O$ (Howell and Gooneratne 1987).

CuO needles and boluses of controlled-release glass containing soluble Cu have also been successfully used to provide a slowly-released Cu supplement from the abomasums or rumen (Suttle and Field 1974, Smart et. al 1992, Allen 1987). The former can be administered in the form of a capsule. The capsule dissolves, releasing the CuO to the stomach and subsequently the abomasums where it dissolves slowly in the acid environment. The latter can be at least twice as effective as the former in terms of the duration of their usefulness. So, whereas Cu needles are effective for up to 6 months, glass would need to be administered once a year. They are however more expensive than the copper needless.

Both the CuO needless and the controlled-release glass of Cu supplementation can be advantageous in avoiding the antagonistic effects of TMs in the GI tract, as also by the perenteral supplementation. This is because, in a highly acidic environment of the abomasum, unabsorbed TM may be degraded and therefore permit the absorption of Cu. However, if these techniques are not further supplemented by increased dietary Cu intake to interact and hence prevent the absorption of any formed TMs whilst in transit from the rumen to abomasums, the absorbed TMs will exert their systemic influence on the absorbed Cu. This may render it unavailable for storage or even when (newly) stored will be removed by TM-albumin (protein) chelators (Quagraine 2002, Quagraine and Reid 2001, Clarke et al. 1987). Caution should however be taken for such combinations of copper supplementation so as to avoid Cu toxicity. These suggestions may also be applicable to any other mode of Cu supplementation that avoids direct access to the rumen content.

"Chelated" Cu supplements

It is necessary to define the term "chelate" carefully, since it is used slightly differently by chemists and by animal nutritionists and the feed industry.

In chemistry, a **complex** is formed when one or more entities or **ligands** containing electron donor (Lewis basic) groups surround an electron-deficient (Lewis acidic) central atom, often a metal ion. A ligand that attaches to a metal ion through only one atom is called a unidentate ("one-toothed") ligand. If it attaches through more than one atom, it is polydentate. In the latter case, the complex is referred to as a **chelate.** (Note that though all chelates are complexes, not all complexes are chelates). The name derives from the Greek Word 'chela', which translates into claw, as an analogy to the clipper-like groups that hold the central metal atom in the ring. Chelates are of particular importance due to their greater stability compared with similar unidentate complexes. This is known as the chelate effect (Schwarzenbach 1952). It enables polydentate ligand to form thermodynamically more stable complexes than analogous unidentate ligands. The chelate effect has both an enthalpy and an entropy contribution (Martell and Hancock 1996). The functional groups in the ligand(s) are located in positions that enable stable ring structures to be formed.

However, the Association of American Feed Control Officials (AAFCO) defines an amino acid metal chelate as the product resulting from the reaction of a metal ion from a soluble metal salt with amino acids with a mole ratio of one mole of metal to one to three (preferably two) moles of amino acids to form co-ordinate covalent bonds. The average weight of the hydrolysed amino acids must be approximately 150 and the resulting molecular weight of the chelate must not exceed 800 (AAFCO 1990). A metal amino acid complex is defined as the product resulting from complexing a soluble metal salt with an amino acid. Since this definition does not consider the actual binding in the complex, it is possible that a complex considered a chelate by animal nutritionists might not be considered as such by a chemist.

To further confuse the issue, various supplements are referred to as chelated minerals, usually in promotional literature, though by either definition they are not. In this paper, the strict chemical definition of chelation will be adhered to. The nutritional use of the word will be placed in quotes, i.e. "chelated". Also the word proteinates will be used as defined by the Association of American Feed Control Officials as the products resulting from "chelation" of a soluble salt with amino acids or partially hydrolysed protein (Kincaid et al. 1986).

"Chelated" minerals have been proposed as superior alternatives to inorganic supplements as they are thought to overcome two of the major limitations of inorganic supplements– low bioavailabilities at low concentrations and toxicity at high levels. Inorganic copper supplements

are absorbed with rates of between 1 and 3 % (Hidiroglou et al. 1990). (Chapman and Bell 1963) report over 80% excretion in cattle of unabsorbed copper from inorganic copper sources.

The increasing utilisation of these supplements centres around the theory that they are more bioavailable, as a result of the stability of the complex, which protects the Cu from further reaction with antagonists that inhibit the absorption of the metal. Many potential advantages have been attributed to the use of "chelated" minerals. Some of these advantages include increased gain, higher reproduction efficiency, and lower levels of mineral requirement. An enhanced immune system and better general access to metabolic systems and tissues are some of the other advantages suggested (Wittenberg 1993, Kincaid 1989, Ho 1977). The major limitation of these products is their higher cost. Although this varies from company to company, they are estimated to cost between six to thirty times the costs of their inorganic counterparts (Patton 1990, Nelson 1988).

Although the mode of action remains largely obscure, there is some evidence to suggest that the chemical form of the metal ingested does indeed affect its bioavailability (Spears 1993, Nelson 1988, Jensen 1979, Ashmead 1991). For organic chelated minerals to be available, presumably they will have to remain intact in the stomach environment, and be delivered intact to the small intestines where much of the absorption takes place. It is not known if thereafter the chelate functions mainly by prevention of precipitation of the metal and releases Cu at epithelial cells, or whether certain complexes are absorbed and transported in tact (Ashmead 1991). Moreover, it is not certain whether the chelated minerals would be able to suppress the influence of such strong antagonists such as TMs in the absorption of copper.

Research comparing the effectiveness of the inorganic and "chelated" mineral supplementation remains inconclusive. Some investigations suggest that "chelated" minerals are superior (Kincaid et al. 1986, Nockels et al. 1993, Du et al. 1993, Du et al. 1994, Debonis and Nockels 1992, Manspeaker et al. 1987). Other reports indicate equal bioavailabilities from "chelated" minerals in comparison to inorganic sources (Kegley and Spears 1994, Ward et al. 1993, Baker et al. 1991, Ward et al. 1996, Luo et al. 1996, Du et al. 1996, Pott et al. 1994, Stansbury et al. 1990, Kincaid et al. 1986, Kincaid 1988, Wittenberg 1990) and even sometimes the inorganic supplements have been found to be superior (Pott et al. 1994, Coffey et al. 1994). Some of the differences were ascribed to differences in age, weight and breed of animals

investigated. The form and sources of the supplements were also different in many of these experiments.

Debonis and Nockels (1992) concluded from an investigation with steers that Cu-lysine was better retained than $CuSO_4$. Similar results were reported by Kincaid et al. (1996). Their findings agree with those of Debonis and Nockels (1992), they found the bioavailability of Cu-proteinate to be greater than that of $CuSO_4$.

Baker et al. (1990) report equal bioavailability for Cu-lysine supplement compared with $CuSO_4$. Ward and Spears (1991) concluded that the two supplements behave similarly in the rumen using an *in vitro* study. In another study, Ward et al (1993) also concluded that the bioavailability of Cu from $CuSO_4$ and Cu-lysine were equal. They used growth, plasma copper and ceruloplasmin activity as indicators of Cu status. Also, Kegley and Spears (1994) found similar bioavailabilities for Cu-lysine and $CuSO_4$ in calves. Similar results were obtained by Wiittenberg et al (1990) who showed that Cu-proteinate was equally bioavailable as $CuSO_4$ in growing steers.

Ellwood (1997) in assessing the availability of copper in steers from Cu-proteinate and Cu(II) oxide, used liver and plasma Cu levels. It was concluded that Cu-proteinate was an effective Cu supplement compared to CuO. CuO has been reported to result in low bioavailability in growing cattle (Kegley & Spears 1994, Langlands et al. 1989) and sheep (Langlands et al. 1989). Relative bioavailabilty of Cu in a Cu-lysine complex for lambs have been estimated to 68% compared with 100% for $CuSO_4.5H_2O$ (Pott et al.1994).

Inconsistencies in the assessment of the efficacies of Cu-"chelates" over $CuSO_4$, is not limited to only ruminants. In an early work by Kirchgessner and Grassman (1970), they found greater bioavailability of Cu with $CuSO_4$ supplements than Cu-amino acid supplements in rats that were deprived of feed, but the reverse was true in the presence of food. In an experiment with pigs, Cu-lysine complex has been found to be as effective as, and in some cases more effective than $CuSO_4$ in enhancing pig performance. However, when the pigs were fed higher concentration (200 mg Kg^{-1}) of Cu in either form, liver Cu concentrations for pigs fed Cu-lysine complex were lower than those fed $CuSO_4$ (Coffey et al. 1994). On the contrary, higher liver Cu concentrations have been observed in pigs fed Cu-lysine than $CuSO_4$ even though the same concentration (200 mg Kg^{-1}) of Cu in both forms were fed to the pigs (Apgar et al. 1995). Zhou et al. (1994) however did not observe any differences in the liver Cu concentrations

between the two Cu sources. Aoyagi and Baker (1993) reported equal Cu availability from commercially available Cu-lysine supplement compared with $CuSO_4$ using chicken bile Cu levels as the response variable. This agrees with another studies with chicks using the liver Cu level to determine the bioavailabilty (Pott et al.1994).

Studies comparing Cu-"chelates" with $CuSO_4$ in the presence of high dietary Mo concentration have also yielded conflicting results. In the absence of high Mo, Cu proteinates were similar in bioavailability to $CuSO_4$. In the presence of high Mo, Cu proteinate A (with low water solubility) appeared to have a greater bioavailability than $CuSO_4$ (Ward et al. 1996). Wittenberg et al. (1990) found no difference in the bioavailabilies of Cu proteinate and $CuSO_4$ in cattle with diet supplemented with ammonium molybdate to increase the Mo content. Kincaid et al. 1986 on the contrary found that Cu proteinate was more bioavailable than $CuSO_4$ in calves fed with hay that contained high concentrations of Mo. In another study, Ward et al (1993) concluded that the bioavailability of Cu from $CuSO_4$ and Cu-lysine is similar in steers in the presence and absence of supplemental Mo and S. The addition of these two supplements decreased soluble Cu to the same extent for $CuSO_4$ and Cu lysine after ruminal fermentation. This finding is consistent with solution chemistry studies where addition of TMs to different Cu-amino acid complexes still results in the formation of the insoluble Cu-TM complex in high ionic strength solutions (Quagraine and Reid 2001, Clarke and Laurie 1982, Laurie et al. 1986).

Research results suggest that there might be certain circumstances where it appears that organic supplements are metabolised differently (Spears 1991, Nockels et al. 1993). It is also not certain if "chelated" supplements stimulate certain biological processes. The inconsistent literature findings regarding the use of these compounds indicate the need for clarification on the apparent advantages of using them, and determining the particular circumstances where they may be beneficial.

Table 1 gives a summary of some bioavailability studies performed in ruminants when some chelated-Cu and $CuSO_4$ supplements are employed in the presence or absence of significant levels of Mo and S. Although not all the vital information required have been provided in the literature to enable exhaustive rationalization of the conditions where either of these supplements appear advantageous, this should be a useful beginning in an attempt to rationalize these findings in the light of the current chemical understanding of Cu(II)-TM-ligand interactions in aqueous media.

Three different conditions are presented in an attempt to rationalize the apparent contradiction of the relative bioavailability of "chelated"-copper and inorganic copper supplements:

1. It appears that in the absence of high Mo and high dietary S or for fasting / copper–depleted animals and when the Cu supplement is such that Cu:Mo ratio is far greater than unity, there seems to be no distinction between the various sources and sometimes the inorganic counterpart is rather more advantageous. This appears so, because the absorption of Cu seem to increase tremendously under deficient conditions, so under conditions where there are no (or no more) interfering substances, "free Cu" or Cu-amino acids can almost equally be absorbed. This seems to be the case for pigs fed with excessive amount of Cu-supplementation and where there is not much distinction between $CuSO_4$ and Cu-amino acid as copper supplements (Zhou et al 1994). In fact, under these conditions, as also applicable to some studies with ruminants (lambs), $CuSO_4$ has sometimes even been found to be more advantageous over the Cu-amino acids. For instance, in the studies by Pott et al. (1993) and Luo et al. (1996) [Table 1] the relative bioavailability of Cu-lysine to $CuSO_4$ were both less than unity, although the latter could statistically be regarded to have the same bioavailability.
2. There seems to be a second condition where there is the presence of significant levels of Cu antagonist (in the diet or rumen), which are not as potent as TMs, but are moderately potent e.g. S^{2-} and L-ascorbate. Under this condition "free" ionic Cu(II) will readily interact with these substances to form products which render the Cu non-bioavailable (for instance as CuS). In the "chelated" form, there may be some "competition" between the ligand (trying to hold on to the Cu) and the antagonist (of moderate potency) for the Cu(II) and hence there may still be, depending on the concentrations and the binding strengths of the competing metal ions and ligands, some Cu in the "chelated" form to be absorbed intact, or transported intact to the epithelial walls of the GI tract to be absorbed subsequently. Under this circumstance, "chelated"-Cu supplements may be more bioavailable than the inorganic counterpart. This may explain why Kirchgessner and Grassman (1970) observed greater bioavailability of Cu with $CuSO_4$ supplements than Cu-amino acid supplements in rats that were deprived of feed, but in the presence of food (likely to contain some interfering substances) they observed the reverse. Also chick

experiments conducted to investigate whether Cu-methionine and Cu-lysine differ from $CuSO_4$ in their reaction to the inhibitory dietary effects of physiological doses of a potent Cu inhibitor, L-cysteine (4000 mg Kg^{-1} diet) or a moderate Cu inhibitor L-ascorbate (1000 mg Kg^{-1} diet), on Cu absorption, affirm the above hypothesis (Aoyagi and Baker 1994). At low Cu doses, and in the presence of significant level of the moderate inhibitor, L-ascorbate (a condition that fits the one under discussion), Cu- methionine and Cu-lysine were better absorbed than $CuSO_4$. However, in the presence of the more potent Cu inbihitor, L-cysteine, there was no significant difference between the absorption from all thee Cu sources when Cu was supplemented at low doses. Furthermore, at high Cu doses and in the presence of the moderate Cu inhibitor, ascorbate (a condition fitting (1) above), $CuSO_4$ was better absorbed, though statistically the difference in absorption with the two Cu-"chelates" was insignificant. Thus, in the presence of moderate doses of Cu supplements and significant levels of less potent Cu-inhibitors like ascorbate, some Cu-amino acids may have slight advantages over $CuSO_4$. This advantage however, would fade with excessive amounts of the supplements, in the absence of significant levels of any inhibitors or in the presence of significant amount of more potent inhibitors like L-cysteine and the notorious TM. In the same line of arguments, high concentrations of even moderately potent antagonist (e.g. ascorbate) can deprive "chelated"-Cu supplements of any advantages over the inorganic counterparts.

3. A third condition is for TM-induced Cu-deficiency (i.e. in the presence of high levels of Mo and S). Under this condition, Cu-amino acids would have no advantage over $CuSO_4$ as already mentioned. The interaction of TMs with different Cu-complexes, especially the amino acid–Cu complexes (Clarke and Laurie 1982, Laurie et al. 1986, Quagraine and Reid 2001, Quagraine 2002) indicates clearly that the presence of these amino acids do not prevent the formation of the insoluble Cu-TMs and subsequently can not improve upon the Cu-absorption. Not even potent chelating agents such as EDTA was found to offer such an advantage (Quagraine and Reid 2001, Quagraine 2002).

Although the definition of "Cu-proteinates" in the nutritional field, includes both Cu-amino acids and Cu-proteins, it is important to distinguish these sources of Cu-chelates hereafter, to understand their individual roles. The use of the term Cu-proteinate thereafter is used in the

strict sense of the complexation between Cu(II) and a protein. "Cu-proteinate" will still be referred to as defined in the nutritional field. Cu-proteinate has been suggested as Cu source that passes through the rumen with little alteration and becomes available for absorption upon exposure to conditions in the abomasums and small intestines (Wittenberg et al. 1990). Although this is possible in the absence of TMs, it does not appear likely in its presence, considering the current understanding of the 3-way interaction between TMs/Cu/albumin(protein) (Wood et al 1987, Clarke et al.1987, Quagraine and Reid 2001). Although these ternary complexes are soluble, Cu in this form is not available, as has been shown to be TCA insoluble in numerous experiments. We also wish to submit that, the generality of this hypothesis to include many other proteins is still inconclusive.

Wittenberg et al. 1990, in using excess Mo and high S (Table 1), observed that both Cu-proteinat and $CuSO_4$ resulted in reduced rumen fluid soluble Mo concentration. This is a clear indication of interaction between all the two Cu supplements and the dietary Mo (TMs) at the rumen level (Wittenberg et al. 1990). The interaction between the Cu-proteinate and the TMs in this case is however not the same as the 3-way interaction between Cu-albumin and TMs, which yields soluble complexes (Wood et al. 1987, Quagraine and Reid 2001). It appears, in this instance, the protein is not involved in the interaction. It would therefore simply be the formation of "Cu-TM" which is insoluble in the high ionic strength fluid of the rumen (Quagraine and Reid 2001). Cu-proteinate which are insoluble in the rumen but soluble in the lower digestive tract may however escape the interaction with TMs and hence be absorbed in the abomasums or the intestines rendering them more bioavailable as observed by Ward et al. (1996) [see also Table 1]. Any advantages of water soluble Cu-proteinates over $CuSO_4$ will only occur under the condition where Mo is absent or better still where dietary Cu:Mo ratio are greater than unity as used by Kincaid et al. 1986 [Table 1] but not when there is excess Mo (and high dietary S) as in the case of the experiment by Wittenberg et al. (1996).

Table 1: Various copper bioavailability studies in ruminants given "chelated"- Cu and $CuSO_4$ supplements (except stated otherwise units for all numerical values are in ppm)

Ruminant	Bioavailability Measurements	Cu supplements	Dietary Cu, Mo, S	Bio-availability	Ref.
Lambs	liver	CS & CL (60-180)	9.5 Cu, 0.9 Mo, 2200 S	CL/CS =0.68	Pott et al. 1993
Lambs	liver	CS & CL (180)	9.8 Cu	CL/CS =0.93	Luo et al. 1996
	Plasma		Cu-depleted	CP = CS	
Steer	Cu solubility in rumen	CP & CS (10)	Cu-depleted, 10 Mo, 3-4,100 S	CP = CS	Wittenberg 1990
			Cu-depleted, 10 Mo, 3-4,100 S	CP = CS	
Steer	Plasma	CL & CS (5)	Low Mo/S	CL=CS	Ward et al. 1993
			5 Mo, 0.2% S	CL=CS	
Calves	Plasma/liver	CP & CS (5-19)	5.6 Mo Cu: Mo (0.9 –2.8)	CP > CS	Kincaid et al. 1986.
		CP & CS (50 mg/day)	Cu-depleted	CP = CS	
Cattle	Plasma/Liver	CP & CS (5)	5 Mo + 0.15% S	CP* > CS	Ward et al. 1996

Definitions: CP = copper proteinate CP* = copper proteinate with poor solubility in water

CL =copper lysine CS = $CuSO_4$

It is necessary to assess other situations or conditions where performances or health response justify the additional cost involved in using them (Spears 1991). At this stage, it appears questionable if the claimed increase in availability assigned to Chelated-Cu over $CuSO_4$ and other inorganic Cu-compounds is justifiable. Furthermore, there appears to be no justification for the large cost differential between these different Cu sources, especially in the treatment of TM-(or Mo/S) induced Cu deficiency. Some researchers suggest that it might be more practical to add an inorganic supplement at a lower cost and lower availability if simple elemental levels are the sole purpose of the supplement (Langlands et al. 1989). With TM-induced Cu deficiency, the critical issue is the choice of the concentration of the supplement to use rather than the source of the supplemention. For Mo-induced Cu-deficiency, it appears that dietary inorganic supplementation such as to provide a dietary Cu:Mo ratio greater than 2 is a reasonable, convenient, cheaper and safer way to go. Under this situation both the pre-absorption and post-absorption roles of TMs will be prevented.

Solubility of Cu-supplements, and how this affects the Cu availability

The solubility of Cu in the rumen is thought to be closely linked to Cu bioavailability. For Cu to be absorbed, it must be in a soluble form. It has been demonstrated that factors that enhance or reduce Cu solubility in the rumen have a similar effect on Cu liver concentration. Cu liver concentration is one of the criteria by which Cu bioavailability is being assessed. There have been a number of studies that attempt to compare the solubility of different Cu sources in the rumen, and attempts to improve the Cu solubility in the rumen.

Ward and spears (1993) concluded that microbial action in the rumen is responsible for the formation of insoluble Cu compounds not normally found in feedstuffs. Ivan et al (1992) found that ruminal protozoa reduces Cu solubility and significantly reduces liver Cu levels, but no effect on Cu solubility in the abomasums (Ivan et al. 1992). Their results are in agreement with a number of reports that have been observed that protozoa decrease copper solubility in the rumen. There could possibly be greater association of copper with the microbial mass in the

rumen (Attaelmannan 1998) or more likely the formation of insoluble CuS or CuTMs (in the presence of Mo) due to increased S^{2-} formation in the rumen.

A number of different methods to influence Cu solubility in the rumen have been attempted. These include the use of ionophores, minerals and antibiotics. Ionophores are a class of compounds that have the ability to bind inorganic cations so as to ease their transport across membrane barriers. They do so by increasing the permeability of the metal to certain ions. Ivan et al. (1992) studied the effect of using supplements of bentonite and monesin, on ruminal Cu solubility. The two supplements were found to affect Cu rumen solubility differently. Rams fed monesin had higher rumen Cu solubility and liver Cu concentration, whereas the bentonite reduced Cu solubility. They forwarded the theory that monesin increases Cu solubility and liver Cu levels by complexing Cu in the rumen, thereby making less Cu available for the formation of insoluble complexes CuS and Cu_2S and subsequently enhancing the transport of the complexed Cu out of the rumen and across the GI tract (Ivan et al. 1992). They however failed to mention that monensin is an antibiotic, and that the increased solubility could be as a result of defaunation of the rumen (Attaelmannan 1998).

The inconsistencies observed in the Cu levels in livers of pigs fed the same amount of Cu-lysine and $CuSO_4$ in two different experiments (*vide supra*) (Coffey et al. 1994, Apgar et al. 1995) have been attributed (Apgar et al. 1995) to the presence of an antibiotic, Aureomycin in one of the experiments (Coffey et al. 1994). The presence of this antibiotic is said to have increased the absorption of $CuSO_4$ more than Cu-lysine. We suggest that, the antibiotic can complex the "free" Cu(II) and be transported better across the membrane walls to the blood. In other words, in the "free" form, Cu(II) permits the ready complexation with the "better" Cu(II) transporter than the amino acid across the borders of the epithelial walls for absorption.

COPPER BIOAVAILABILITY AND COPPER SPECIATION

Because of the multiplicity of factors that control the distribution of metal ions, ligands and their complexes in different biological fluids, it is not possible to easily predict in which specific fluid compartment they will be found. The distribution of metal ions into different fractions has physiological implications; this distribution helps specify the functions to be performed by the metal ion, and helps regulate the metal's homeostasis.

Bioavailability is often defined as the proportion of an ingested nutrient that is absorbed and, more importantly, utilized for metabolic processes (Fox et al. 1981). Although the concept of bioavailability is straight forward, the problems arise in its determination as numerated below. Because utilization can be quite difficult to quantify, other authors are less restrictive and define bioavailability as the proportion which is absorbed and can be utilized by the animal (Bender 1989). Liver Cu deposition would fit the latter definition, but not the former (Pott et al. 1994). Accumulation of Cu in liver has been used as the primary criterion of Cu bioavailability of supplemental sources of ruminants (see Table 1) and other animals like chicks (Pott et al. 1994). The use of the traditional balance studies to assess Cu bioavailability is complicated by Cu absorption and retention values that are low relative to those other elements and by the interactions between Cu and other dietary constituents. The only Cu radioisotope readily available commercially, ^{64}Cu, has a half-life of 12.9 h, rendering it unsuitable for many types of studies, especially involving ruminants (Pott et al. 1994). Plasma Cu concentration as well as soluble Cu in the intestinal tract are also usually used as a criteria to assess the bioavailability of Cu from the various Cu sources from the diet of an animal (see Table 1). Cu levels in many other tissues are also sometimes used; for instance bile Cu concentration in Chicks have been used for such assessments (Aoyagi et al. 1993). For the above, it is mostly the total concentration of Cu which is determined in the various organs or the various biological fluid compartment.

The determination of total concentrations though useful, is not sufficient for environmental and clinical considerations. It is indeed essential in many analytical schemes. Toxicologists have long recognised that the chemical form of an element greatly influences its role in vivo. Small changes in the form or the oxidation state of a metal have been known to produce dramatic changes. A classic example is that of mercury. The toxic effects associated with mercury result mostly from methyl mercury as compared to elemental mercury or inorganic mercury salts (Reid 1981). It is indeed, the concentration of the individual copper species, rather than the total concentration which are the determinants of the metal's biological significance, deficiency and toxicity in a given situation.

Once a ruminant ingests copper, the metal undergoes innumerable interactions. For instance, the influence of several minerals such as iron (Fe), cadmium (Cd), zinc(Zn), nickel (Ni), lead (Pb) and selenium (Se) in preventing Cu absorption are well known (Underwood 1977,

Gooneratne et al. 1989) although the interrelations are complex and poorly understood (Gawthorne 1987). Despite the complexities, competition for protein sites by various metals with Cu is a contributing factor. To fully understand the effects of these interactions between Cu(II) ions, other metal ions and the ligands they encounter, it is essential to know the precise mixture of chemical forms present in a biofluid. This is the **speciation,** which has been defined as "the individual physico-chemical forms of an element which together makeup the total concentration in a sample" (Kumar et al. 1994). Speciation analysis is particularly tedious for trace elements in biological systems, as the total amounts are at or close to the limits of detection of present day analytical instrumentation, whereas speciation analysis requires sub-totals. In addition, biological systems are complicated matrices that could be difficult to calibrate.

It is quite often not possible to experimentally determine the speciation of Cu as the number of potential ligands that are capable of binding copper *in vivo* is very large. In addition, the separation of the individual complexes is often impossible, and where possible quite tedious. Changes in any of the biological conditions could disturb the steady state or equilibrium position attained, and renders the measured situation not truly representative of that which was originally present. It should also be borne in mind that due to the high lability of Cu complexes the fragile equilibrium established could be distorted by the very act of measurement.

COPPER SPECIATION IN BIOFLUIDS BY COMPUTER MODELLING

In the absence of experimental data for speciation, resort is made to computer models. Computer modelling attempts to imitate but simplify reality by stressing those aspects that are assumed to be important and omitting those considered to be non-essential. The widespread use of computers has enabled the use of more sophisticated mathematical models that provide a useful picture of the distribution of metal ions. It also provides insights into the effects of excesses and deficiencies as well as allowing for variations in different ligand concentrations. The real benefits of computer models are the predictive ability built into them. Such models are useful in determining the predominant ligands to which the metal is bound.

Many computer programs have been used to study the equilibria in a variety of biological fluids, notable the distribution in blood plasma. One of the early models of blood plasma was that of Perrin and Aggarawal (1973). A more comprehensive study was undertaken

by May (1976) using the program ECCLES. He published data for the distribution of Cu (II) and Zn(II) ions in an enlarged blood plasma and latter expanded the program to include other metal ions and ligands (May et al. 1977). These programs were widely adopted for a number of studies by Berthon et al. (1978, 1980, 1984, 1986). Models for exogenous metal ions in blood plasma have also been published (Cole et al. 1985). Copper speciation in the fish gill microenvironment at various levels of pH have also been reported using the computer program MINEQUA2 (Tao et al. 2001 and 2002).

Copper speciation in ruminant fluids by computer modeling

Computer simulations to determine the fate of Cu(II)-lysine complex prior to arriving at the rumen has been reported (Attaelmannan and Reid 1996). The speciation of the Cu(II) lysine system was determined in McDougall's solution (McDougall 1948), a simulated form of bovine saliva as shown in Table 2.

Table 2: The composition of McDougall's Solution

Constituent	Concentration (M)
$NaHCO_3$	1.67×10^{-1}
Na_2HPO_4	2.61×10^{-2}
KCl	8.04×10^{-3}
NaCl	8.56×10^{-3}
$MgSO_4$	4.06×10^{-4}
$CaCl_2$	3.60×10^{-4}

Using the concentration of the constituents of this solution and the formation constants of the different complexes as input values, a simulation was performed to determine the distribution of copper in McDougall's solution at physiologically relevant pH values. The pH range covered was from 6.5 to 9.0. Ruminant saliva is usually slightly alkaline, but rarely exceeds a pH of 8.

The concentration of copper used for the model was 3×10^{-5} **M**, a modest value for the nutritional requirements of a healthy cow (McDonald et al. 1988).

The outcomes of the speciation calculations are depicted in Figures 1 and 2 .

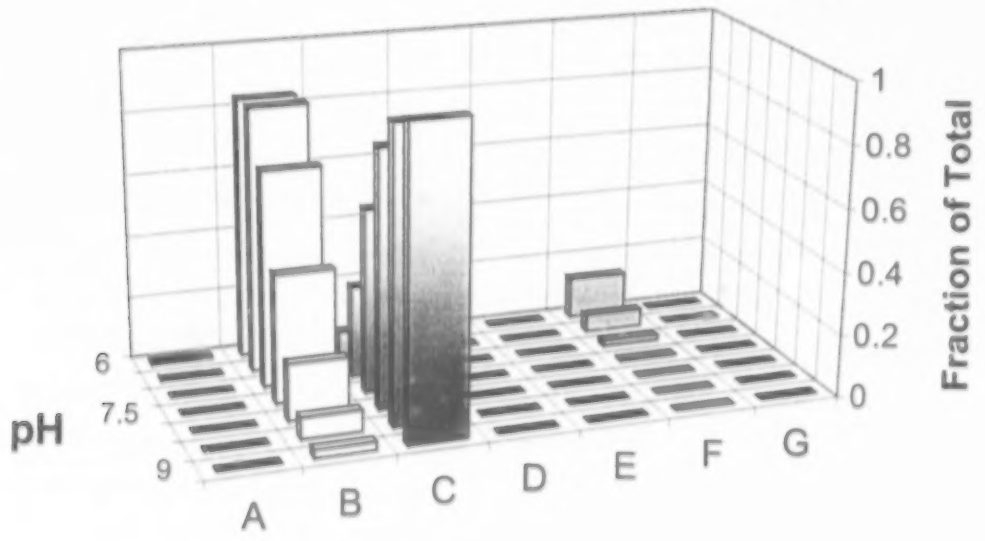

Figure 1 Copper speciation in McDougall's Solution as a function of pH using an inorganic supplement at a copper concentration of 3×10^{-5} **M** (Attaelmannan and Reid 1996)

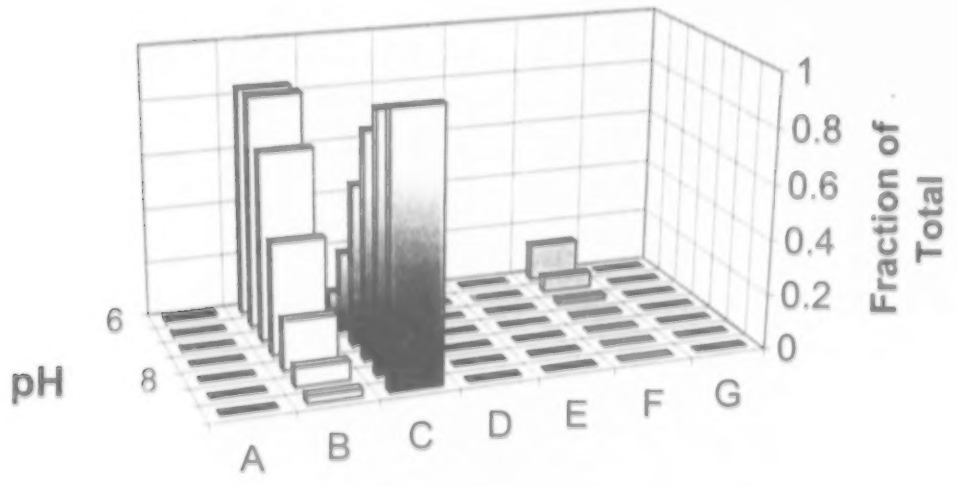

Figure 2 Copper speciation in McDougall's Solution as a function of pH using a copper lysine supplement. At a total lysine concentration of 6×10^{-5} **M** and a copper concentration of

3×10^{-5} **M** (Attaelmannan and Reid 1996)

Key:

A : Free Cu^{2+} ; **B :** Cu^{2+} in 1:1 carbonate complexes. ; **C :** Cu^{2+} in 1:2 carbonate complexes. ; **D :** Cu^{2+} in 1:1 lysine complexes. ; **E :** Cu^{2+} in 1:2 lysine complexes. ; **F :** Cu^{2+} in 1:1 phosphate complexes. **; G :** Cu^{2+} in all other complexes.

Figure 1 shows the copper speciation when the copper is introduced as an inorganic salt, while Figure 2 shows the speciation when copper is introduced as a 1:2 copper lysine mixture. The similarity is striking, suggesting that the addition of lysine does not significantly alter the speciation of copper. One can immediately see that in both cases the copper is found predominantly as the 1:1 copper carbonate complex. These results indicate that although lysine complexes copper quite strongly, at equilibrium, in the rumen lysine-complexed copper will be in the minority, owing to the significantly higher quantities of the other ligands. The concentration of ionic copper at all levels is found to be very low, accounting for less than one percent of the total amount of available copper over the entire pH range investigated. This very small amount of free copper decreased with increasing pH values. This is because of the high affinity of copper ions for co-ordinating ligands. This affinity quite often makes it impossible to measure the concentration of free ionic copper in biological media. For instance, May et al. (1977) estimate the concentration of ionic copper in blood plasma to be of the order of 10^{-20} mol/L, a level that is well beyond the detection limit of analytical instrumentation. The results, in summary, suggest that if Cu is administered as Cu-lysine "chelate", this will disintegrate and the Cu will be present mainly as carbonate and phosphate complexes, as also for free (inorganic) Cu.

The conclusion from the study discussed above has been supplemented and extended using ^{1}H NMR experiments (Reid and Attaelmannan 1998). The chemical shift changes of the lysine protons (e.g C^{γ} as in Figure 3) as a function of Cu concentration was monitored. Armed with a knowledge of the chemical shifts from previous experiments (Reid and Attaelmannan 1998), and the distribution data from the computer model (Attaelmannan and Reid 1996), it was possible to predict what the lysine chemical shifts in these spectra should be. As shown in Figure 3, a good agreement between the predicted and the experimental values were achieved, confirming the previous conclusion (Attaelmannan and Reid 1996) that the bulk of Cu(II) does not remain in the lysine form in McDougall's solution, thus confirming the reliability of the computer model. The scope of the computer model has been expanded in the latter study to include the major low molecular mass (LMM) ligands in the rumen constituents of a rumen sample as an undertaken to determine the likely fate of Cu in rumen liquor in the presence and the absence of lysine (Reid and Attaelmannan 1998). The low molecular weight substances included in the model are the volatile fatty acids (VFA), acetic, propanioc, and butanioc acids together with the gases ammonia, methane, hydrogen sulfide and carbon dioxide

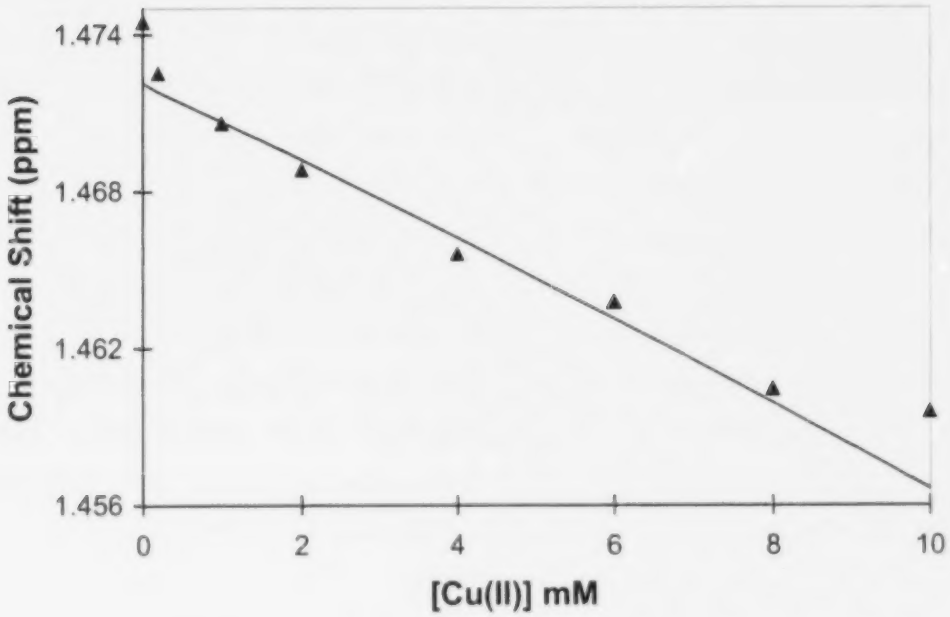

Figure 3: Changes in lysine C^{γ} proton chemical shift as a function of concentration in McDougall's solution. Symbols represent experimental data while line represents values calculated from computer modeling.

Table 2: Values of ligand concentrations used in rumen model

Ligand	**Concentration** (mM)
Chloride	22
Acetate	80
Propionate	15
Butyrate	10
i-Butyrate	1
Valerate	0.9
i-Valerate	0.7
PO_4	20
HCO_3	21
CO_2	36

Ammonia 11

TABLE 3: Stepwise formation constant (log k) data used in the simulation for anticipated complexes of stoichiometric formula $[M_pL_qH_r]/[M]^p[L]^q[H]^r$.

System	p	q	r	log β	Ref.
Acetic Acid	0	1	1	4.74	a
Copper Acetic Acid	1	1	0	1.60	a
	1	2	0	1.1	
	1	3	0	0.3	
	1	4	0	-0.1	
Propanoic Acid	0	1	1	4.68	a
Copper Propanoic Acid	1	1	0	1.70	a
	1	2	0	0.92	
Butanoic Acid	0	1	1	4.62	a
Copper Butanoic Acid		1	1	0 1.75	a
	1	2	0	0.95	
iso-Butanoic Acid	0	1	1	4.62	a
Copper iso-Butanoic Acid	1	1	0	1.75	estimate
	1	2	0	0.95	
Valeric Acid	0	1	1	4.62	a
Copper Valerate System	1	1	0	1.75	a
	1	2	0	0.95	
Phosphoric Acid	0	1	1	1.70	a
	0	1	2	6.46	a
	0	1	3	10.79	
The Copper Phosphate	1	1	0	3.3	a
	1	1	1	1.3	
Carbonic Acid	0	1	1	6.02	a
	0	1	2	9.57	
The Copper Carbonate	1	1	0	6.75	a
	1	2	0	3.17	
Ammonia	0	1	-1	9.14	a
Copper Ammonia	1	1	1	4.12	a
	1	2	0	3.51	
	1	3	0	2.88	
	1	4	0	2.09	

Table 2: Continued.....

System	p	q	r	log β	Ref.
Lysine	0	1	1	10.529	b
	0	1	2	19.658	
	0	1	3	21.914	
Copper (II) Lysine	1	1	1	18.81	b
	1	2	2	35.90	
	1	2	1	26.41	
	1	2	0	15.73	
	1	1	2	23.58	

Ref a = Martell and Smith 1982
Ref b = Berthon 1995.

(Attaelmannan 1998). The analytical concentrations of these ligands used in the model (see Table 2) were arrived at after exhaustive comparisons of various values in the literature (Church 1988, Van Soest 1994, Hungate 1966 and Haresign and Cole 1981). The formation constants used in setting up the expanded model also is outlined in Table 3.

The speciation calculations were performed under variable concentrations of VFA's to reflect their known variations in rumen fluids due to differences in diet (Church 1988). However, this did not result in any significant changes in the overall distribution of Cu(II). As illustrated in Figure 4, the results from the computer simulation indicate that the bulk of the copper, in addition to being present as carbonate and phosphate complexes as in saliva (Attaelmannan and Reid 1996), would be present mainly as complexes with ammonia. The copper ammonia complexes are however not electrically neutral and would thus be difficult to be absorbed in that form. The speciation of copper was not altered when copper was introduced into the model as a copper lysine supplement. From these results it can be conclusively asserted that the final distribution of copper is effectively identical regardless of the form in which the metal is administered. It was discussed earlier (*vide supra*) that if the supplement is introduced as a copper lysine supplement it would disintegrate prior to its arrival at the rumen. The results in the rumen indicate that the complex would not be reconstituted in the rumen.

^{1}H NMR studies was also carried out in actual rumen liquor (ultrafiltered) to monitor the chemical shift of VFA's with varying Cu(II) levels in the presence or absence of lysine, to properly understand the fate of copper-lysine in rumen liquor and also validate the rumen computer model as reported (Reid and Attaelmannan 1998, Attaelmannan 1998). The acetate methyl protons, being the most intense resonance in the rumen liquor was chosen to monitor

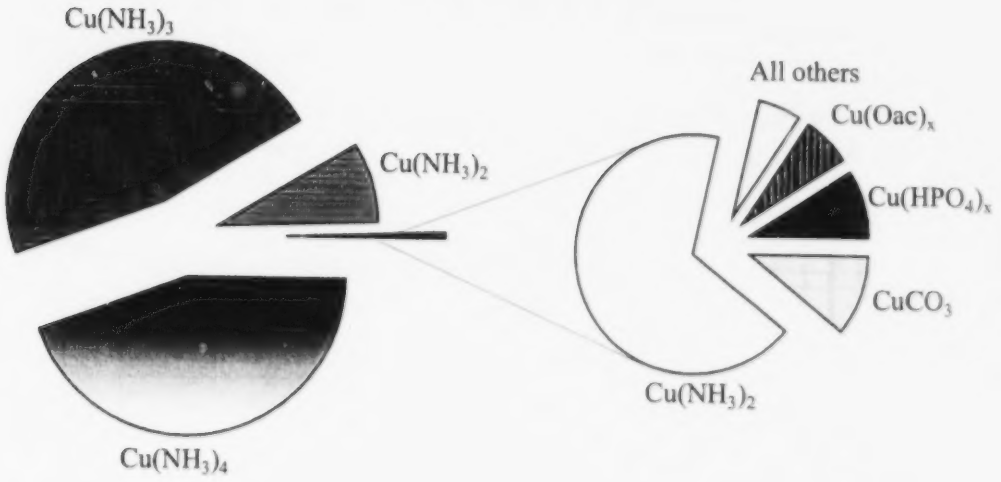

Figure 4: Copper speciation in rumen with LMM ligands at a pH 6.0.

the copper speciation. From calculated chemical shifts of the different acetate species as well as the copper(II) acetate complexes, and using the computer simulation of the copper speciation, it was possible to predict the chemical shift changes that was to be expected from the addition of different amount of copper (II) solution to the rumen liquor.

The calculated chemical shifts of acetate as compared to the measured values in the rumen liquor in the absence or presence of varying amounts of lysine are shown in Figure 5. The computer model predicted that the addition of 0.8 m**M** lysine would not alter the speciation significantly; the bulk of the copper would still be complexed to ammonia. When

actual NMR experiments were conducted this was also the case. The model closely predicted the observed changes in chemical shift (Figure 5c). However, with the addition of 4.0 mM lysine (see Figure 5d) the acetate chemical shift was altered significantly, indicating that the acetate was being stripped off the Cu(II). The acetate was even further stripped of the metal with the addition of 20 mM lysine (Figure 5e). However, discrepancies were observed between the experimental and the predicted values at these stages, with the extent of the discrepancy becoming more

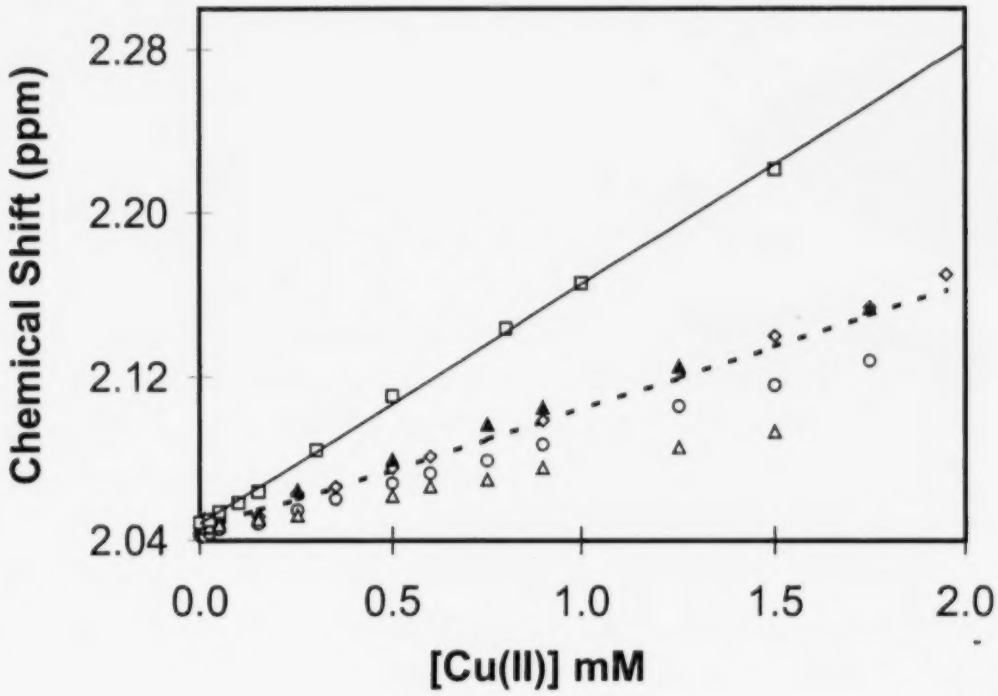

Figure 5: Acetate proton chemical shifts in different media (a) • aqueous media (b) ▲ LMM rumen liquor (c) ◇LMM rumen liquor with 0.8mM lysine added (d) ○ LMM rumen liquor with 4.0mM lysine added (e) △ LMM rumen liquor with 20mM lysine added.

as a function of copper(II) concentration in the simulated rumen fluid. In the presence higher concentrations of lysine, the model predicts that the bulk of the copper will be present as copper-lysine complexes in contrast to the observed. In other words, the model seemed to have

predicted a lower amount of Cu0acetate complexes than that actually observed. The lack of agreement between the two could not be explained adequately in that study (Reid and Attaelmannan 1998, Attaelmannan 1998).

Recent expansion of the speciation modeling of copper(II) ions in the bovine rumen model in our laboratory to include the possible formation of mi l ligand complexes (Essilfie-Dughan and Reid 2003) has offered explanation to the discrepancies observed previously. The introduction of mixed ligand species (copper-amino-acetate, copper-propanoate-acetate, copper-lysinate-acetate) and their formation constants, estimated from 1:1 copper complex formation constants (Sigel 1973) were included into the computer model in succession. The mixed ligand complex formation involving acetate and lysine when introduced into the computer model, gave a very good fit to the experimental data as shown in Figure 6.

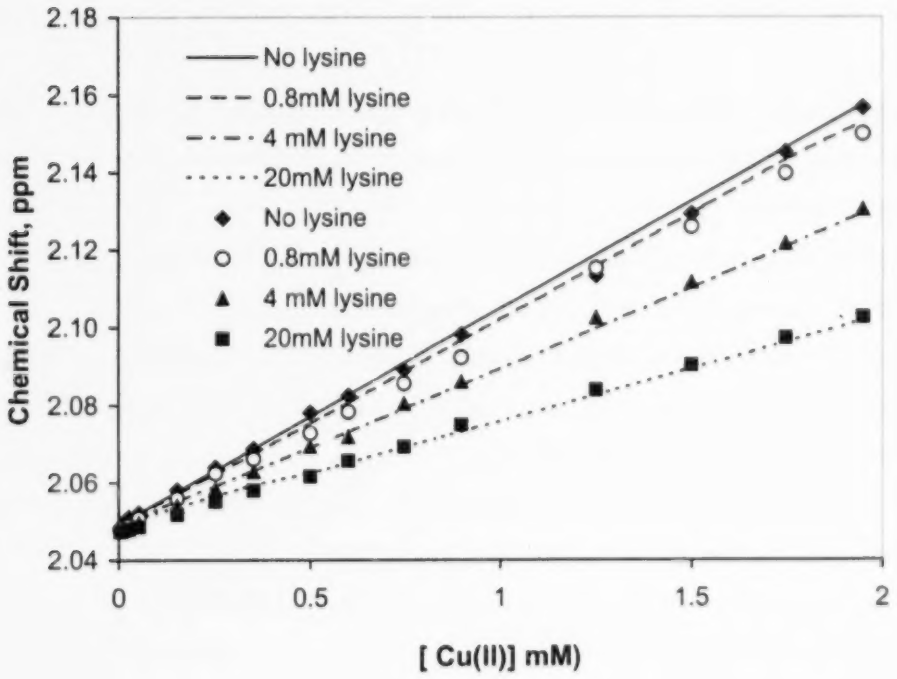

Figure 6: Acetate proton chemical shifts in LMM rumen liquor as a function of the added copper(II) concentration when lysine is absent or present at different amounts. Points represent experimental data, while curves represent model predictions.

The implication is that, when lysine is introduced into the rumen fluid, it is unable to strip off the acetate but rather form mixed ligand complex with it. The absence of the mixed ligand complex in the previous model is the reason for the discrepancy, as discussed earlier.

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